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THE EFFECT OF SUBCUTANEOUS INJECTIONS OF TRYPSIN ON THE BLOOD-SUGAR AND ON INSULIN ACTION.

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II. BLOOD-SUGAR EFFECT OF TRYPSIN ABOLISHED BY A PRELIMINARY INJECTION OF PARTIALLY INACTIVATED FERMENT.

PREVIOUS work has shown (Buckley, 1931) that the subcutaneous injection of Fairchild's glycerinated trypsin into rabbits is followed in the majority of cases by an immediate rise and fall of blood-sugar level, and that the extent of the rise can be modified by altering the degree of activity of the ferment. It was further pointed out that the condition of the rabbit for a few days after the injection of trypsin was similar to that of a diabetic individual suffering from some infection in that, during this period, insulin failed to lower the blood-sugar in the usual way. This similarity lent support to the theory (Rosenthal and Behrendt, 1926) that one factor concerned in the inhibition of the proper action of insulin in those diabetics who are suffering from an infection is the presence of a proteolytic ferment in the inflammatory exudate.

The following experiments show that though a rabbit which is given repeated doses of trypsin responds with a greater rise and fall of blood-sugar level with each injection, it is possible to abolish these blood-sugar changes by giving a preliminary dose of weakened ferment. Presumably the latter promotes the formation of antitrypsin in the body of the rabbit, and the antibody thus produced prevents a subsequent dose of active trypsin from causing its usual reaction.

Methods.

Male rabbits were used as the experimental animals. Their food consisted of a daily ration of oats and cabbage, but no food was given on the day of a test until the experimental proceedings were over. The animal had had no food, therefore, for about 17 hours before any blood-sugar estimation.

The blood-sugar level and rectal temperature were first obtained. Then a subcutaneous injection of trypsin was given ; and subsequent estimations of

TABLE I.—*Effect of Repeated Doses of Trypsin on the Blood-sugar (mgm. per 100 c.c.) and Body Temperature (Fahr.).*

Rabbit.	Date.	Injection.	Before.		½ hour.		1 hour.		1½ hours.		2 hours.		Maximum change.	
			B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.
31	2.ii.28	0·1 c.c. trypsin	118	102·1°	119	101·5°	114	101·3°	160	101·2°	197	102·0°	+79	−0·9°
	20.ii.28	0·1 ,, ,,	134	101·2°	248	100·0°	265	99·8°	..	..	221	101·0°	+131	−1·4°
36	17.ii.28	0·1 c.c. trypsin	104	100·3°	175	99·6°	152	99·2°	143	100·1°	131	100·4°	+71	−1·1°
	1.v.28	0·1 ,, ,,	112	102·5°	103	99·8°	132	99·4°	225	98·3°	147	98·8°	+113	−4·2°
	3.v.28	0·1 ,, ,, *	134	102·4°	274	98·2°	166	96·0°	207	96·0°	156	96·2°	+140	−6·4°
42	13.ix.28	0·1 c.c. trypsin	123	102·2°	138	100·8°	150	100·6°	131	101·0°	137	101·0°	+27	−1·6°
	26.ix.28	0·1 ,, ,,	84	102·4°	118	101·6°	218	100·0°	157	98·6°	235	99·3°	+151	−3·8°
	27.ix.28	0·1 ,, ,, *	106	102·8°	84	101·0°	165	100·8°	..	99·8°	89	97·6°	+59	−5·2°

* 1 unit of insulin given at the same time.

TABLE II.—*Blood-sugar (mgm. per 100 c.c.) and Temperature (Fahr.) Changes due to Trypsin, following*

TABLE II.—*Blood-sugar (mgm. per 100 c.c.) and Temperature (Fahr.) Changes due to Trypsin, following a Preliminary Injection of Heated Trypsin.*

Rabbit.	Date.	Injection.	Before.		½ hour.		1 hour.		1½ hours.		2 hours.		3 hours.		Maximum change.	
			B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.
36	1.v.28	0·1 c.c. trypsin	112	102·5°	103	99·8°	132	99·4°	228	98·3°	147	98·8°	..	..	+113	−4·2°
	3.x.28	0·1 ,, ,, heated to 56° C. for 30 min.	91	102·2°	106	101·6°	..	101·8°	116	101·6°	120	101·7°	..	..	+29	−0·6°
	9.x.28	0·1 c.c. trypsin	129	103·0°	143	101·8°	137	101·6°	136	102·0°	125	102·0°	..	..	+14	−1·4°
38	25.vi.28	0·05 c.c. trypsin	167	102·5°	175	100·8°	204	100·2°	189	100·5°	164	101·0°	157	101·2°	+37	−2·3°
	29.vi.28	0·05 ,, ,, heated to 56° C. for 10 min.	119	103·2°	127	103·1°	127	102·8°	127	103·0°	121	103·0°	122	103·5°	+8	−0·4°
	9.vii.28	0·05 c.c. trypsin heated to 56° C. for 30 min.	112	102·0°	123	101·6°	116	101·6°	108	101·7°	128	101·2°	123	101·8°	+16	−0·8°
	12.vii.28	0·5 c.c. trypsin 1 unit insulin	142	103·7°	129	103·4°	103	103·1°	82	101·8°	37	100·7°	65	101·0°	−105	−2·0°
	13.vii.28	0·1 c.c. trypsin	119	103·8°	115	103·8°	116	102·3°	116	101·3°	116	101·9°	124	102·4°	+8	−2·5°
37	18.vii.28	0·5 c.c. trypsin heated to 56° C. for 10 min. 1 unit insulin	113	103·1°	92	102·8°	72	102·6°	77	102·3°	75	102·0°	97	101·2°	−41	−1·9°
	26.vii.28	0·5 c.c. trypsin 1 unit insulin	126	102·8°	86	102·9°	83	102·4°	72	102·4°	64	101·2°	75	101·4°	−62	−1·6°
	30.iv.29	0·1 c.c. trypsin	117	102·6°	102	102·4°	110	102·3°	106	101·8°	106	102·4°	..	..	−11	−0·8°
43	22.x.28	0·1 c.c. trypsin heated to 56° C. for 44 hrs. 1 unit insulin	115	102·4°	95	102·0°	50	101·7°	59	102·0°	53	101·8°	..	..	−65	−0·7°
	30.x.28	0·1 c.c. trypsin	92	102·6°	102	102·4°	114	102·6°	109	102·6°	..	..	..	..	+22	−0·2°
44	6.xi.28	0·1 c.c. trypsin heated to 56° C. for 46 hrs.	110	102·4°	109	101·9°	125	101·6°	109	101·7°	116	101·3°	..	..	+15	−1·1°
	13.xi.28	0·1 c.c. trypsin	118	103·8°	118	102·0°	121	102·5°	112	102·8°	121	103·4°	..	..	+3	−1·8°
47	15.iv.29	0·1 c.c. trypsin heated to 56° C. for 48 hrs.	115	102·5°	117	102·2°	119	101·9°	125	101·7°	..	..	..	..	+10	−0·8°
	17.iv.29	0·1 c.c. trypsin heated to 56° C. for 48 hrs. 1 unit insulin	127	102·6°	116	99·9°	62	100·9°	67	101·8°	60	102·2°	..	..	−67	−2·7°
	7.v.29	0·1 c.c. trypsin	108	102·4°	106	101·4°	108	101·7°	102	101·6°	104	102·0°	..	..	−6	−1·0°

the blood-sugar were made at half-hourly intervals for 2 or 3 hours. The blood was obtained by heart puncture and the sugar content estimated by Maclean's method. The rectal temperature was taken at the time of each blood-sugar test.

Fairchild's sterile glycerinated trypsin was used. It was diluted in the ratio of 1 part to 10 saline and injected subcutaneously into the flank.

Results.

Table I shows the increasing effect on the blood-sugar of repeated doses of active trypsin.

Table II shows that this effect is abolished if a preliminary dose of partially inactivated trypsin is given—that is, trypsin which has been heated to 56° C.; and that while repeated doses of active trypsin tend to produce a marked constitutional effect, shown by a sharp fall in body temperature, this also is abolished by a preliminary dose of weakened ferment.

III. THE EFFECT OF PARTIALLY INACTIVATED TRYPSIN ON THE DEVELOPMENT OF THE INSULIN-RESISTANT STATE.

It has been shown above that when trypsin, which has been heated to 56° C., is injected, subsequent doses of active trypsin do not produce the immediate rise and fall of blood-sugar level nor the sharp depressions of body temperature which otherwise occur in increasing strength after repeated doses of active ferment. The results suggest that the weakened ferment has promoted the formation of sufficient antitrypsin to antagonize the usual effects of trypsin.

It was therefore possible to imagine that the second effect of trypsin, namely the inhibitory effect that it has on insulin action for a few days subsequent to the trypsin injection, might also be abolished by a previous dose of heated ferment. The following experiments represent an attempt to show whether this is the case.

Methods.

A few days after a preliminary dose of partially inactivated trypsin, that is, trypsin which had been heated to 56° C. for varying periods of time, an injection of active trypsin was given. This, as shown above, had little or no power to affect the blood-sugar level. Immediately following this dose of active trypsin, a daily injection of one unit of insulin was given, the blood-sugar being estimated before and at half-hourly intervals after the injection for 2 hours.

Other conditions of the experiment were the same as those described above.

In the case of Rabbit 36 a preliminary injection of heated trypsin was given, after which a dose of active trypsin failed to produce its usual blood-sugar effect. However, for the next three days the inhibitory effect on insulin action was as complete as it had ever been after an injection of active trypsin.

Results.

TABLE III.—*Absence of Immediate Effect of Trypsin on the Blood-sugar Level, but Persistence of Inhibitory Effect on Insulin Action on Subsequent Days.*

Rabbit.	Date.	Injection.	Before.		½ hour.		1 hour.		1½ hours.		2 hours.		Maximum change.	
			B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.	B.S.	Temp.
36	30.iv.28	1 unit insulin	117	102·0°	121	101·5°	115	100·9°	56	100·8°	40	100·4°	−77	−1·6°
	3.x.28	0·1 c.c. trypsin heated to 56° for 30 min.	..	..	..	..	..	..	..	..	..	..	..	..
	9.x.28	0·1 c.c. trypsin	129	103·0°	143	101·8°	137	101·6°	136	102·0°	125	102·0°	+14	−1·4°
	10.x.28	1 unit insulin	128	103·2°	131	102·9°	117	102·6°	129	102·8°	121	102·0°	−11	−1·2°
	11.x.28	1 ,, ,,	117	102·8°	115	102·6°	104	102·2°	100	102·2°	..	..	−17*	−0·4°
	12.x.28	1 ,, ,,	91	103·0°	..	..	78	102·9°	..	..	117	103·0°	−13	−0·1°
37	17.vii.28	1 unit insulin	106	102·9°	84	102·6°	57	102·6°	45	102·1°	45	101·8°	−61	−1·1°
	18.vii.28	0·05 c.c. trypsin heated to 56° for 10 min.	..	..	..	..	..	..	..	..	..	..	..	..
	26.vii.28	0·05 c.c. trypsin	126	102·8°	86	102·9°	83	102·4°	72	102·4°	64	101·2°	−62	−1·6°
44	27.vii.28	1 unit insulin 1 ,, ,,	103	103·3°	144	101·6°	67	100·9°	77	100·5°	67	100·8°	−36	−2·5°
	5.xi.28	1 unit insulin	127	101·0°	102	101·0°	75	100·7°	44	100·8°	26	100·9°	−101	−0·3°
	6.xi.28	0·1 c.c. trypsin heated to 56° for 30 min.	..	..	..	..	..	..	..	..	..	..	..	..
	13.xi.28	0·1 c.c. trypsin	118	103·8°	118	102·0°	121	102·5°	112	102·8°	121	103·4°	+3	−1·8°
	14.xi.28	1 unit insulin	117	102·8°	103	102·6°	109	102·6°	93	102·6°	95	102·5°	−24	−0·3°
	15.xi.28	1 ,, ,,	107	102·6°	..	102·5°	50	102·5°	41	102·4°	..	..	−66*	−0·2°
47	12.iv.29	1 unit insulin	116	102·0°	78	101·8°	69	101·1°	50	100·9°	49	100·8°	−67	−1·2°
	15.iv.29	0·1 c.c. trypsin heated to 56° for 48 hours	..	..	..	..	..	..	..	..	..	..	..	..
	7.v.29	0·1 c.c. trypsin	108	102·4°	106	101·4°	108	101·7°	102	101·6°	104	101·2°	−6	−1·2°
	8.v.29	1 unit insulin	125	102·8°	115	102·5°	103	102·4°	107	102·4°	107	..	−22	−0·4°
	10.v.29	1 ,, ,,	130	101·8°	91	102·1°	..	..	55	102·3°	50	102·2°	−80	+0·5°

* The maximum fall of B.S. would probably have been greater if an estimation had been made at 2 hours.

In the case of Rabbit 37 a preliminary dose of heated trypsin was given, after which a dose of active trypsin was inadequate to counter-balance the blood-sugar effect of a dose of insulin given at the same time; that is, the immediate effect of trypsin on the blood-sugar was absent. But the inhibitory effect on insulin action on the following day was present to some extent.

Rabbits 44 and 47 also show the absence of the immediate effect of trypsin on the blood-sugar, after a preliminary dose of weakened ferment; while the inhibitory effect on insulin action on the following day is present in each case.

CONCLUSIONS.

1. A rabbit which is given repeated injections of trypsin will, on each occasion, react with a greater rise and fall of blood-sugar level.

2. If a preliminary dose of partially inactivated trypsin be given, subsequent injections of active trypsin do not produce the usual rise of blood-sugar level.

3. While repeated doses of trypsin tend to produce a generalized disturbance, exemplified by a marked fall in body temperature, when a dose of heated trypsin has been given, a subsequent injection of active ferment does not produce this effect.

4. A subcutaneous injection of trypsin has also an inhibitory effect on the action of insulin on subsequent days; but this effect was not abolished by a preliminary dose of weakened ferment.

5. Thus it was not found possible to prevent the insulin-resistant state by injections of partially inactivated trypsin. Further experiments are needed, however, to show whether, with modifications of dosage and time interval between injections, such a protection may not yet be achieved.

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THE EFFECT OF SUBCUTANEOUS INJECTIONS OF TRYPSIN ON THE RESPIRATORY METABOLISM OF THE DOG.

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THE underlying pathological process responsible for the state of insulin resistance, which develops in diabetic patients with infective conditions, has been suggested by some to consist in a direct neutralization of insulin in the blood-stream by proteolytic ferments derived from inflammatory exudates (Rosenthal and Behrendt, 1926).

Others think that, in certain pathological conditions, trypsin may pass from the acinar tissue of the pancreas direct into the blood-stream, instead of by the pancreatic duct, and there act as an antagonist to insulin in two ways, first, by direct neutralization, and second, by increasing glycogenolysis in the liver (Epstein and Rosenthal, 1925).

It has also been suggested that the infective process acts by stimulating the reactive mechanism of the thyroid and adrenal glands, both of which are known antagonists to insulin action (Cramer, 1926; Lawrence and Buckley, 1927).

To test this last hypothesis an investigation was made consisting in an accurate study of the basal metabolic rate before, and after, the subcutaneous injection of trypsin, as this would indicate any disturbance of the thyroid control.

METHOD.

It has been found in the rabbit that a subcutaneous injection of trypsin produces a condition of resistance to insulin action similar to that which is present in a diabetic patient suffering from an infection, and since trypsin can be injected in accurate amounts, it was a convenient material with which to produce the insulin-resistant state in this experiment. The dog was used as the experimental animal, since a high degree of accuracy could be depended on with this animal, using the technique for basal metabolic rate determinations of the department in which facilities for this work were given (Boothby and Sandiford, 1920; Kitchen, 1924). The dog weighed 14·5 kg. It had been thoroughly trained for work in respiratory metabolism—a training occupying many weeks, and consisting in making the animal gradually accustomed to the following conditions:

At 8.30 a.m. the dog was laid on its right side on a wooden rack; the front and back legs were gently, but firmly, tied to the slats of the rack, and a

broad strip of calico was passed over the middle of its body to bind it down without force. A conical mask was then fitted over the head and bound on to the neck with strips of thin rubber, the neck being previously shaved in order to prevent any possibility of air-leaks. Inlet and outlet air-tubes were fixed into the mask, which thus communicated with the outside air and with a Tissot respirometer respectively. A thermometer and the end-piece of a stethoscope were placed under the animal's flank, to enable temperature and pulse-rate readings to be taken during the tests. Finally a glass cover was let down over the dog, as a protection against disturbance from noise and draughts. The temperature of the chamber was kept constant by an electric bulb inside the cover.

The dog remained quiet, contented and relaxed in this position until 12.30 p.m., during which time a series of estimations of metabolic rate could be carried through.

No food was given on any experimental day until the tests were over, the interval between metabolism tests and the preceding meal being approximately 21 hours ; the specific dynamic action of food was thus avoided. After a preliminary rest period of half an hour in the experimental chamber to allow the metabolism to stabilize at a basal level, expired air was collected in the Tissot respirometer over frequent 10-minute periods ; each was analysed by Haldane's method and the metabolic rate calculated therefrom. About twelve such tests, with intervals of five minutes, were carried through during the morning. There was very little variation in the results, and the average was taken as the basal rate for that day.

In Table I, (*a*) shows a period of $3\frac{1}{2}$ months during which estimations of the normal basal metabolic rate were made from time to time, with no other experimental procedure. The rate was very constant, the total calories per hour varying only from 20·2 to 20·9, except for one occasion on which they reached 21·5. The respiratory quotient lay between 0·75 and 0·79.

On a subsequent day 0·7 c.c. Fairchild's glycerinated trypsin, diluted to 7 c.c. with normal saline, was injected subcutaneously into the loose tissues in the groin of the right hind leg. This dose of trypsin was reckoned to be the equivalent for the dog, weight for weight, to that which inhibits insulin action in the rabbit (Buckley, 1931).

Table I (*b*) shows the series of tests done on the morning on which the trypsin was given. Six preliminary 10-minute periods showed that the basal metabolism for that day lay between 20·5 and 21·4 calories per hour, the respiratory quotient between 0·73 and 0·77. Then the injection was given. The dog immediately became restless, and no air analysis was therefore made for three-quarters of an hour. At the end of this period the total calories reckoned per hour were 23·5, and the respiratory quotient 0·81. During the next hour the metabolic rate fell to 21·3 calories per hour, and the respiratory quotient to 0·78. The restlessness caused by the injection took about $1\frac{1}{2}$ hours to disappear completely. The pulse-rate was not increased, but the respiration-rate was almost doubled. This resulted in a greater output of CO_2, but the O_2 intake being also increased, the respiratory quotient was only slightly raised. The rectal temperature, at the end of the morning's tests, was 100·4°, the usual temperature ranging from 99·6° to 100·2°.

TABLE I.—*The Effect of a Subcutaneous Injection of Trypsin on the Respiratory Metabolism of the Dog.*

(a) *Control Estimations:*

Date.	R.Q.	O_2 litres per hr.	CO_2 litres per hr.	Calories—total per hr.	Pulse per min.	Respirations per min.	Weight in kilogrammes.	Rectal temperature (Fahrenheit.)	Remarks.
27.viii.29	0·77	4·37	3·36	20·8	76	26	13·8	100·0°	Quiet.
5.ix.29	0·76	4·32	3·28	20·5	72	24	13·9	100·0°	,,
27.ix.29	0·79	4·50	3·56	21·5	78	26	14·3	100·3°	,,
8.x.29	0·78	4·33	3·38	20·7	73	27	14·1	99·7°	,,
15.x.29	0·76	4·27	3·25	20·3	73	26	14·3	100·0°	,,
30.x.29	0·76	4·32	3·28	20·6	76	26	14·4	100·2°	,,
5.xi.29	0·75	4·26	3·20	20·2	73	19	14·3	99·7°	,,
13.xi.29	0·76	4·28	3·25	20·4	67	23	14·5	99·6°	,,
27.xi.29	0·78	4·37	3·41	20·9	71	21	14·4	100·2°	,,
16.xii.29	0·75	4·40	3·26	20·9	68	24	14·9	..	,,

(b) *Effect of the Subcutaneous Injection of 0·7 c.c. Fairchild's Glycerinated Trypsin Diluted to 7 c.c. with Normal Saline:*

16.xii.29 Hour.									
8.45–8.55	0·73	4·42	3·23	20·8	73	20	14·9	..	Quiet.
9.0–9.10	0·76	4·38	3·33	20·8	68	23	..	..	,,
9.15–9.25	0·75	4·42	3·32	20·9	66	26	..	..	,,
9.30–9.40	0·75	4·32	3·24	20·5	66	28	..	..	,,
9.45–9.55	0·76	4·51	3·43	21·4	70	23	..	..	,,
10.0–10.10	0·77	4·35	3·35	20·7	65	24	..	..	,,
10.25–	Subcutaneous injection of trypsin.								
11.15–11.25	0·81	4·88	3·95	23·5	63	28	..	..	Very restless.
11.35–11.45	0·83	4·80	3·98	23·3	65	45	..	..	Restless.
12.11–12.21	0·78	4·45	3·47	21·3	66	34	..	..	Quiet.
12.29–12.39	0·78	4·58	3·57	21·9	68	25	..	100·4°	,,

(c) *Estimations made on Succeeding Days after the Injection of Trypsin:*

17.xii.29	0·78	4·59	3·58	21·9	77	25	14·9	100·6°	Slightly restless.
18.xii.29	0·81	4·22	3·42	20·3	71	27	14·9	99·9°	Quiet.
19.xii.29	0·75	4·54	3·41	21·5	72	26	14·9	100·0°	,,

On the following day (Table I (*c*)) the morning's tests gave an average metabolic rate of 21·9 calories per hour and a respiratory quotient of 0·78—both somewhat higher than normal. The temperature was 100·6° and the dog still showed some slight restlessness.

On the second day after the injection of trypsin normal conditions again prevailed.

It is interesting to compare these results with those obtained by Wilhelmj and Boothby on the effect of intravenous injections of thyroxine on the respiratory metabolism of the dog (Wilhelmj and Boothby, 1930). The curve of extra heat production after 10 mgrm. thyroxine is more marked and much more sustained than that obtained with trypsin, nor was restlessness present as a complicating factor. The basal metabolic rate begins to rise within 20 hours from the time of the injection and reaches its peak in 22 to 70 hours, the return to the normal level being more gradual. The whole curve of extra heat production covers a period of about 8 days. During the same period the pulse-rate is quickened, the maximum increase being at the time of the peak of extra heat production.

SUMMARY.

Within an hour after the subcutaneous injection of 0·7 c.c. trypsin solution, the metabolic rate of the dog rose from 20·7 to 23·5 calories per hour, and the respiratory quotient from 0·77 to 0·81. The animal was, however, hyperexcitable and restless for about an hour after the injection, and then gradually became quiet. The respiration-rate was nearly doubled, but the pulse-rate remained unaltered.

After $1\frac{3}{4}$ hours a normal metabolic rate had again been reached, though the respiration-rate was still somewhat quickened and the respiratory quotient slightly raised.

Twenty-four hours after the trypsin injection the metabolism was again very slightly above the normal basal level and the temperature slightly raised. But on this day, also, the dog was slightly hyperexcitable and restless. On the second day following the injection conditions were again normal.

The restlessness and increased respiratory movements which followed the administration of trypsin undoubtedly increased the metabolism, and the increase actually found was of an order compatible with the restlessness. It was therefore unlikely that any of the extra heat production was due to stimulation of the thyroid gland and increase of its thyroxine output. The fact that the pulse-rate remained slow throughout the experiment supports this view.

The experiments of Wilhelmj and Boothby on the effect of 10 mgm. thyroxine on the respiratory metabolism of the dog are quoted for comparison.

CONCLUSION.

An experiment is described in which it is shown that trypsin in a dose comparable to that which, in the rabbit, produces the condition of insulin-resistance, has no noticeable effect on the thyroxine output of the dog, as measured by the respiratory metabolism.

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THE RELATIONSHIP BETWEEN THE VIRUSES OF ZOSTER AND VARICELLA AS DEMONSTRATED BY THE COMPLEMENT-FIXATION REACTION.

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IT is now forty-two years since von Bokay first suggested that varicella and some cases of zoster were caused by the same infective agent. Von Bokay's thesis rested on clinical evidence, more particularly on the observation that susceptible children frequently developed varicella after exposure to a case of zoster, the interval between exposure and the development of varicella corresponding with the incubation period of that disease. Numerous workers have confirmed von Bokay's observation. Bruusgaard (1932) gives a good summary of this evidence. Of the various associations of zoster and varicella which can occur, that of zoster followed by varicella in susceptible contacts within the incubation period of the latter disease alone occurs with sufficient regularity to exclude its being due to chance; this was drawn attention to by Ker (1920). There is, however, some clinical evidence which, at first sight, at any rate, does not support the common ætiology of these two diseases. The age and seasonal incidence of zoster and varicella are different. Varicella does not necessarily protect against zoster, and *vice versâ*. Further it has been reported that in certain isolated communities zoster may be common and varicella unknown. Investigation of this problem by direct biological experiment has been delayed by the lack of a suitable experimental animal, but experiments on children have been carried out. Kundratitz (1925, *a*, *b*) produced a vesicular lesion in 17 out of 28 children with zoster vesicle fluid, and in three of these positive cases a generalized eruption indistinguishable clinically and histologically from varicella occurred. Several healthy contacts developed clinical varicella after

the usual incubation period. Further, children who had had varicella were insusceptible to the inoculation of zoster vesicle fluid, and those which had recovered from experimental zoster infection were found to be immune to the inoculation of varicella vesicle fluid. Bruusgaard (1932) has repeated these experiments with zoster vesicle fluid and obtained similar results, and in one instance he achieved passage producing clinical varicella in a healthy child by inoculation from a primary positive vesicle. Netter and his colleagues in France have approached the problem from a different direction. Using the crusts of zoster and varicella lesions as antigens they have investigated the sera of convalescents for specific antibodies by means of the complement-fixation reaction. Their findings (Netter and Urbain, 1931) suggest that all cases of zoster, both idiopathic and symptomatic, and varicella are due to the same virus. Lauda and Silberstern (1925) questioned the specificity of these findings, but Bedson and Bland (1929), using a somewhat different technique from Netter's, found that zoster convalescent serum contained a specific antibody. Their observations were, however, limited to three cases. More recently, Thomsen (1932) reported results supporting Lauda and Silberstern, but as details of his technique are not available it is impossible to criticize or account for his results. Thus it will be seen that although subsequent work has tended to confirm the thesis of von Bokay, some of the evidence is conflicting. Rivers and Eldridge (1929), for instance, found that only one out of three zoster sera neutralized varicella virus. It was therefore decided to re-investigate the question from the serological aspect, as did Netter.

EXPERIMENTAL DATA.

The technique of the complement-fixation reaction employed is that described by Bedson and Bland (1929). Inactivated convalescent serum 0·1 c.c., antigen 0·1 c.c. and complement 0·1 c.c. (2 M.H.D.) were mixed in that order and the volume made up to 0·8 c.c. with saline. After 2–3 hours at room temperature 0·2 c.c. sensitized sheep's cells were added and the whole incubated at 37° C. for half an hour. Readings were then taken, and again after standing in the refrigerator overnight.

Antigens.

Specific.—Two types of antigens were tried. The one, as used by Netter, consisted of a saline suspension of the dried crusts from older lesions, the other being a dilution of vesicle fluid in phosphate buffer. The former proved so inferior in our hands that its use was soon discontinued. It was frequently anticomplementary, and when diluted sufficiently to overcome this defect it was poor in fixing power. In Netter's investigations complement was titrated in the presence of the antigen, and the quantitative method of complement-fixation of Calmette-Massol was used. Ether extraction of the dried crusts was tried to see if in that way a more satisfactory crust antigen could be obtained, but without success. The vesicle fluid was used as a rule in a dilution of 1/20, but its antigenic potency was found to vary from case to case, and an occasional sample required to be used in higher concentration. Rarely a vesicle fluid was found to be useless as an antigen. The majority of vesicle fluids collected in

this investigation were culturally sterile. Out of 17 zoster vesicle fluids 14 gave no bacterial growth, and of the 3 contaminated ones, 2 were satisfactory antigens giving specific fixation.

Cases of varicella from which a sufficiency of vesicle fluid could be obtained were rarely encountered. Out of 4 such cases one provided a vesicle fluid which was useless as an antigen.

The anticomplementary activity of these antigens was invariably tested before use by titrating them in the presence of 1 M.H.D. of complement. An antigen was considered satisfactory if it did not inhibit the action of 1 M.H.D. of complement when present in twice the concentration used in the test. It was found, however, that the anticomplementary activity of some samples could be removed without damage to their antigenic potency by heating them for 10 minutes at 56° C.

TABLE I.—*Complement-Fixation Reaction with Zoster Vesicle Fluid and Zoster and Varicella Convalescent Sera.*

Fixation : 2¼ hours at room temperature. Complement : 2 M.H.D.

Serum.	Patient's No.	Antigens. Zoster vesicle fluid 1–20.	Dermatitis herpetiformis vesicle fluid 1–20.	Saline.
Convalescent zoster (3 weeks)	43	++++	—	—
		++++	—	—
Zoster (9 days)	45	—	—	—
		—	—	—
Zoster (3 weeks)	45	++++	—	—
		+++±	—	—
Convalescent chicken-pox (3 weeks)	13	++++	—	—
		++++	—	—
Ditto	16	++++	—	—
		++++	—	—
,,	27	++++	—	—
		+++±	—	—
Control	69	—	—	—
		—	—	—
,,	70	±	±	±
		—	—	—
,,	71	—	—	—
		—	—	—
Saline	..	—	—	—
		—	—	—

The degree of fixation of complement in this and subsequent tables is indicated by the signs ++++, representing complete fixation. The upper row is the reading after 30 minutes at 37° C., and the lower one after standing overnight in a refrigerator.

TABLE II.—*Complement Fixation Reaction with Varicella Vesicle Fluid and Zoster and Varicella Convalescent Sera.*

Fixation : 3 hours at room temperature. Complement : 2 M.H.D.

Serum.	Patient's No.	Antigens. Varicella vesicle fluid 1–20.	Dermatitis herpetiformis vesicle fluid 1–20.	Saline.
Convalescent chicken-pox	21	++++	−	−
		++++	−	−
,, ,,	22	++++	−	−
		++++	−	−
,, ,,	24	+++±	+	−
		+++	−	−
Convalescent zoster	28	++++	−	−
		++++	−	−
,, ,,	30	++++	−	−
		++++	−	−
,, ,,	32	++++	−	−
		++++	−	−
Control	49	+	+	+
		±	±	−
,,	50	−	−	−
		−	−	−
,,	51	−	−	−
		−	−	−
Saline	..	−	−	−
		−	−	−

TABLE III.—*Crossed Fixation Reaction.*

Fixation : $2\frac{1}{2}$ hours at room temperature. Complement : 2 M.H.D.

Serum.	Patient's No.	Antigens. Zoster vesicle fluid 1–20.	Varicella vesicle fluid 1–25.	Dermatitis herpetiformis vesicle fluid 1–20.	Saline
Convalescent zoster	47	++++	++	−	−
		++	−		
,, ,,	53	++++	++++	±	±
		+++±	+	−	−
Convalescent chicken-pox	27	++++	+++±	−	−
		++++	+		
Saline	..	−	−	−	−
		−	−	−	−

N.B.—Although in this test the chicken-pox antigen was rather more dilute than the zoster antigen, it has been noted on other occasions that chicken-pox antigen did not as a rule give such good fixation as zoster antigens either with homologous or heterologous sera.

TABLE IV.

Sera.		Individual cases. Antigens. Zoster.	Individual cases. Antigens. Varicella.	Total tests. Antigens. Zoster.	Total tests. Antigens. Varicella.
Zoster	Number tested	44	21	84	34
	Complete fixation	42	16	77	26
	Partial fixation	0	5	4	8
	Negative	2	0	3	0
Varicella	Number tested	17	10	38	10
	Complete fixation	16	7	26	7
	Partial fixation	1	3	9	3
	Negative	0	0	3	0
Control	Number tested	80	17	111	19
	Complete fixation	1	0	1	0
	Partial fixation	0	0	0	0
	Negative	79	17	110	19

Control.—In the majority of tests the bullous fluid from 5 cases of dermatitis herpetiformis diluted 1 in 20 was used as the control antigen. Other control antigens used were the diluted vesicular fluid from a case of herpes febrilis, a suspension of psoriatic scales and three samples of cerebro-spinal fluid. In all 10 different antigens were employed, and since the results obtained with them were consistently negative they will not be referred to further.

Sera.—The sera investigated were obtained from cases of zoster and varicella either when the disease was still active or, much more frequently, about the third week of convalescence. Although zoster antibodies were found in one serum collected two days after the appearance of the eruption, as a rule they were not present in readily detectable amounts until the end of the second week of the disease. Netter and Urbain (1920) found that the antibody concerned in this reaction was in feeble concentration during the first few days of disease, reached a maximum during the second week, and might still be present 18 months after recovery. They reported specific fixation with sera obtained 3, 5, 6, 8, 9, 11, 12 and 18 months after recovery from zoster and after 8 months in a case of varicella. In our series antibody was found 10 and 15 months after recovery from zoster and 2 months after varicella. Control sera were obtained from normal individuals, and from patients suffering from diabetes mellitus, psoriasis, dermatitis herpetiformis and various cardiac and renal conditions. The sera were heated for 20 minutes at 56° C. on the day of the test.

RESULTS.

Tables I, II and III are typical examples of the findings obtained. The first shows specific fixation given by zoster and varicella sera with a zoster antigen, whilst the second shows a similar test with the exception that a varicella antigen was used in the place of a zoster antigen. Owing to the

difficulty of obtaining samples of both zoster and varicella antigens at the same time, it was only occasionally that a double test with both antigens and sera could be put up. An example of such a double test is given in Table III. In Table IV the findings of this investigation are summarized. The term "complete fixation" indicates that there was no hæmolysis after 30 minutes at 37° C., and little or no hæmolysis at the second reading after standing overnight in the refrigerator. In those results classified as "partial fixation" there was slight hæmolysis at the first but still some fixation at the second. The results are classified as "individual cases" and also under the heading "total tests", since many of these sera were tested more than once using different antigens, and in some instances more than one sample of serum was examined from the same patient. It will be seen from this table that zoster and varicella convalescent sera consistently give cross-fixation—in fact the results with the heterologous antigens are as good as those with the homologous one. With the exception of one case of zoster which gave negative readings with serum collected on the 9th day of the disease but positive fixation with serum taken on the 28th day, and three varicella sera which gave negative results with one zoster antigen (a poor one) and positive reactions when re-tested with a different antigen, these duplicate tests have given concordant results. Of the negative reactions given by two zoster sera with zoster antigen, one was collected on the 6th day from a case with a very scanty eruption, whilst the other, collected 14 days after the onset of disease, gave complete fixation with a varicella antigen. Presumably in the latter case the zoster antigen used was a poor one, but owing to the impossibility of repeating the test this can only be surmised, and the result is returned as negative. It is evident, however, that the antigenic property of individual vesicle fluids varies considerably. Reference has already been made to the observation that the varicella vesicle fluids were definitely poorer antigens than the zoster vesicle fluids. This is brought out by the figures in Table IV, for the percentage of partial fixations is definitely greater with the varicella antigens, whether the serum is homologus or heterologous.

The control serum which gave positive fixation with a zoster antigen came from a woman of 25 who had had varicella in childhood. The reason for this reaction is not obvious.

CONCLUSIONS.

1. The serum of individuals convalescent from zoster and varicella contains specific antibodies, which can be demonstrated by complement fixation using the vesicle fluids as antigens.

2. Zoster vesicle fluid gives equally good fixation in the presence of both zoster and varicella sera, and the same is true of varicella vesicle fluid.

3. These findings are in conformity with those of Netter and his colleagues.

4. They indicate the close relationship and possible identity of the viruses of zoster and varicella.

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THE FAILURE OF PROLONGED ADMINISTRATION OF IRON TO CAUSE HÆMOCHROMATOSIS.

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THE production of hæmochromatosis was a secondary objective in previous experiments concerned with the storage of iron following its administration by various routes (Polson, 1928, 1929, *a*, 1929, *b*). No satisfactory evidence was then obtained to show that excess of iron in the liver caused cirrhosis, although the longer experiments lasted from one to one and a half years. In the present experiments a high iron content of the liver was maintained in rabbits over periods from 1–4 years by means of repeated administration of iron subcutaneously and intra-peritoneally. The absence of cirrhosis in all of the rabbits suggests that the cirrhosis observed in human hæmochromatosis is not the result of siderosis *per se*. This has been much debated from time to time, and Muir (1925) voiced the current opinion when he stated that "there seems to be no doubt that the cirrhosis precedes the deposit of pigment". Dunn (1921) instanced three cases of hæmochromatosis. In two of these interstitial pancreatitis was accompanied by siderosis; in the third "the cirrhosis of the pancreas was more marked . . . but no iron was present". Cappell (1930) found no evidence of hæmochromatosis in the organs of mice and rats given large doses of iron when under experiment for as long as 14 months.

EXPERIMENTAL PROCEDURE.

Twenty-six rabbits received dialysed iron (B.D.H. Ltd.) in doses of 5 c.c. and 10 c.c. subcutaneously and intra-peritoneally at intervals during the course of experiments which lasted from 1–3 years. These experiments permitted observation of the degree and progress of iron storage in the liver.

A further ten rabbits were similarly injected and were under experiment from 37–46 months ; the details of experiment are shown in Table I.

TABLE I.—*Protocols of Rabbits Given Iron Subcutaneously and Intraperitoneally at Intervals during 27–46 months.*

Rabbit.	Duration of experiment in months.	Dosage of iron in c.c.				Total dose of iron in c.c.		Interval between last dose and death.	% of iron in liver, dry weight.
		1st year.	2nd year.	3rd year.	4th year.	S.C.	I.P.		
290*	27	30	5	10	0	15	30	1 week	4·65
192†	29	22	20	0	0	25	17	13 months	2·90
272†	30	40	10	0	0	25	25	18 ,,	1·99
193†	31	22	15	20	0	35	22	2 ,,	6·95
188†	33	32	20	20	0	35	37	2 ,,	6·67
276‡	37	30	5	30	20	35	50	1 month	4·87
287†	37	25	5	30	20	35	45	1 ,,	3·53
183‡	38	30	15	30	0	45	35	6 months	3·43
189‡	38	27	20	30	0	45	32	7 ,,	4·26
274‡	42	25	5	30	40	35	65	2 ,,	4·21
275‡	42	25	5	30	40	35	65	2 ,,	6·07
281‡	44	35	10	30	40	45	70	4 ,,	7·67
286‡	44	25	5	30	50	35	75	1 month	6·95
190*	46	20	20	30	40	45	65	4 months	6·87
194*	46	20	20	30	40	45	65	4 ,,	4·09

* Ill when killed. † Died. ‡ In fair health when killed.

In each experiment a post-mortem examination was followed by histological examination of selected tissues, in order to demonstrate the degree of iron storage and the presence or absence of fibrosis of the liver and pancreas. The iron content of the livers of five rabbits under experiment from 27–33 months and of the ten rabbits under experiment from 37–46 months was estimated by the method of Fowweather (1926) ; these results are shown in Table I.

The galactose tolerance test, as modified by Polson (1933), was performed on six rabbits when the experiments had lasted 35–38 months, and repeated on four rabbits at the end of 44–46 months.

EXPERIMENTAL OBSERVATIONS.

(A) *Rabbits Under Experiment from 1–3 years.*

The normal iron content of the liver of the rabbit was estimated as 0·036 per cent. dry weight (Fowweather and Polson, 1930). The livers of five rabbits under experiment from 27–33 months contained from 1·99 to 6·95 per cent.

(average 4·63 per cent.) of iron dry weight. The rabbit whose liver contained 1·99 per cent. had had none for 18 months. While the precise amount was not estimated in the remaining twenty-one rabbits, it was possible to judge by histological examination that the siderosis was severe, and comparable with that of the livers submitted to chemical examination. Large aggregations of iron within endothelial cells were situated within and around the portal tracts. A quantity of iron was also stored in the liver-cells, especially those of the peripheral zone of the lobules. Iron granules were present in the Kupffer cells, and the liver-cells of the mid- and central zones were stained diffusely when treated with potassium ferrocyanide and hydrochloric acid. Cirrhosis of the liver was absent from each rabbit.

(B) *Rabbits Under Experiment from* 37–46 *months.*

The liver iron content of these rabbits ranged from 3·43 to 7·67 per cent. (average 5·19 per cent.) dry weight. The iron content of two livers from cases of human hæmochromatosis was estimated as 2·92 and 3·34 per cent. dry weight respectively.

Histological examination confirmed the severity of the siderosis. The aggregations of iron within endothelial cells were a striking feature, and in rabbit 281 these aggregations were approximately one-third the diameter of a liver lobule. Not only were they larger than those seen in rabbits under experiment for shorter periods, but they were also more numerous. Greater iron storage in the liver-cells was also observed, and the majority contained granules of iron. The peripheral zone of the lobules was particularly affected, and there the cells were packed with coarse iron granules. Iron was also present in the Kupffer cells. Notwithstanding the severity of the siderosis there was no evidence of liver cirrhosis and the pancreas was at all times healthy.

Rabbit 183 differed from the other nine in that the presence of a nodule of hyperplasia associated with localized liver fibrosis suggested a positive result. The nodule measured approximately $\frac{3}{4}$ in. in diameter, and was prominent because its pale rust colour contrasted sharply with the deep rust colour of the remainder of the liver. It was situated in the upper part of the right lobe close to the junction between the right and left lobes, and it projected somewhat from the surface. Histological examination showed that the nodule was composed of islands of hyperplastic liver-cells enclosed within coarse strands of fibrous tissue ; the liver-cells were necrotic in one area. The remainder of the liver contained 3·43 per cent. dry weight of iron, and although precise determination was not made on tissue from the nodule, it was apparent on histological examination that there was much less iron in the nodule than in the remainder of the liver. It is probable that this was an intercurrent lesion, and it is unlikely that experimental treatment played any part in its production. This was the only fibrotic change seen in a series of thirty-six livers, and the nine other rabbits, under experiment for a similar period, had a greater siderosis and yet no liver fibrosis.

The galactose tolerance tests were negative on the six rabbits examined at 35–38 months, and at 44–46 months two were negative, one positive and one probably positive.

DISCUSSION.

Polson (1929,*a*) showed that the administration of a single dose of 20 c.c. of dialysed iron to three rabbits subcutaneously had raised the liver iron content to 1·43, 1·49 and 2·25 per cent. dry weight respectively when they were killed at 7–8 months later. In the present experiments the maintenance of a high liver iron content was demonstrated in rabbits under experiment from 1–3 years. The rabbits under experiment from 3–4 years received similar doses of iron at corresponding periods during the first 3 years. The further doses of iron given during the fourth year could not alone account for the finding of from 3·42 to 7·67 per cent. of iron in the livers at death. It is therefore evident that the liver iron content was maintained at a high level throughout the 3–4 years of experiment. The siderosis produced in rabbits was much greater than that observed in the two cases of human hæmochromatosis, where the liver iron content was 2·93 and 3·42 per cent. dry weight. This observation was supported by histological evidence. Yet liver and pancreatic damage was absent in the rabbits. [The pancreas of one case of human hæmochromatosis contained but a trace of iron, although an advanced interstitial pancreatitis was present.] It is shown that in rabbits an excess of iron in the body over long periods caused neither liver cirrhosis nor hæmochromatosis. Nor was it possible to detect any impairment of liver function, even though the majority of the liver-cells contained a heavy charge of iron. These results, and the confirmation of Dunn's observation in respect of the human pancreatic fibrosis, suggest that siderosis *per se* plays no part in the production of the characteristic hepatic and pancreatic lesions of human hæmochromatosis.

SUMMARY.

The liver iron content of thirty-six adult rabbits was maintained at a high level from 1–4 years. The terminal level in ten rabbits under experiment from 3–4 years ranged from 3·42 to 7·67, and averaged 5·19 per cent. dry weight. Liver cirrhosis and pancreatic damage was absent from all. It is therefore shown that excess of iron in the body over long periods caused neither liver cirrhosis nor hæmochromatosis in rabbits, and it is unlikely that excess of iron is responsible for the hepatic and pancreatic lesions of human hæmochromatosis.

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THE INFLUENCE OF MALTOSE ON THE GROWTH AND TOXIN PRODUCTION OF *C. DIPHTHERIÆ.*

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IT has recently been shown by Pope and Llewellyn Smith (1932) that media can be prepared which give consistently culture filtrates of high Lf value. In this medium maltose is used as one of the energy sources, and is largely responsible for the enhanced toxin production obtained.

In the present paper we give the results of further experimental work which has been carried out to find the optimal concentration of maltose for toxin production.

For the first series of experiments we measured the rate of toxin production in medium containing varying quantities of maltose, but later, in addition to measuring the toxin, we also measured the total bacillary growth. A knowledge of this latter factor enables us to express the results obtained in the ratio toxin produced/growth produced, and thus determine variation between growth and toxin for media of different composition.

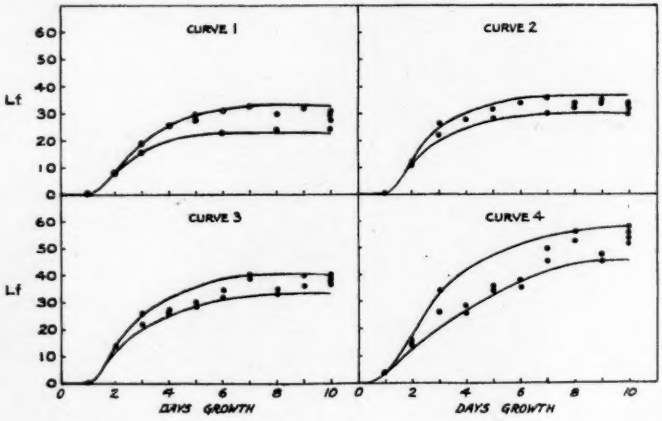
EXPERIMENTAL.

A batch of medium was prepared as described by Pope and Llewellyn Smith from beef muscle ; it was subdivided into four portions, to which were added 0·1, 0·2, 0·4 and 0·8 per cent. maltose. Each portion was filtered through a Seitz filter into 1000 c.c. capacity bottles (200 c.c. of medium into each bottle), and sterilization completed by steaming for 10 minutes at 100° C. The bottles of medium were placed horizontally in an incubator at 34° C., and inoculated with a portion of film which had grown for 24 hours on similar medium. Two bottles were removed daily for each concentration of maltose until the ninth day, and the remainder of the bottles on the tenth day. Each culture was treated with toluol and later filtered, the amount of toxin in each filtrate being determined by the flocculation method of Ramon (1922).

The results obtained are shown in Curves 1–4, from which it will be seen that the amount of toxin produced increased with increase in the maltose concentration. The curves are drawn in each case to fit the highest and lowest toxin values found, since the values of the culture filtrates from different bottles were seldom exactly the same on any day. The irregularity in toxin production is due to the fact that each bottle must be considered as an individual which received a varying amount of inoculum, and also to the fact that throughout

growth portions of the film are continually falling to the bottom of the medium ; thus during the growth period the amount of diphtheria bacilli in the film varies considerably. We have found that growth and toxin production occur only in the film.

In further experiments it was decided to compare growth and toxin production. Methods devized for estimating the amount of bacterial growth fall roughly into three groups : (*a*) Methods involving counting the number of organisms microscopically (see Wilson, 1922), (*b*) estimation of dry weight using micro-analysis methods (Coombs and Stephenson, 1926), and (*c*) comparison of the opacity of bacterial suspensions either against standard known suspensions (Dreyer and Gardner, 1916) or against barium sulphate standards (Brown and Kirwan, 1915). For various reasons we have used a method based on the opacity of suspensions. In this work the organism being studied



CURVES 1–4.

grows as a film on the surface of the medium, and in order to take a sample from any particular culture it is necessary to reduce the whole to a fairly uniform state. This can be accomplished by shaking the culture with glass beads, and although various cultures differ markedly in the ease with which they can be emulsified, we have found that with care a number of samples can be removed from a culture each of which give the same results with narrow limits. For the determination of the opacity we have used a photo-electric colorimeter, of which a brief description is given below. As an arbitrary standard we have chosen a suspension of such strength that a layer 1 cm. thick will reduce the transmission of light to 50 per cent. of that given by a similar layer of distilled water, and this we term standard or " S ". A calibration curve was prepared as follows : An emulsion of *C. diphtheriæ* was prepared which reduced the transmission of light to 25 per cent., and from this we prepared known dilutions and measured the transmission given by each dilution. The results when plotted (logarithm percentage transmission against concentration of organisms) gave a straight line provided the percentage transmission was between 30 and 70

per cent. It was therefore possible to prepare emulsions of such strength that the reading lay between these values, and calculate their value either as a multiple or fraction of " S ", since—

$$\frac{E_1}{E_2} = \frac{C_1}{C_2} \text{ or } C_2 = C_1 \frac{E_2}{E_1},$$

where E_1 = — logarithm per cent. transmission (here taken as 50 per cent. transmission).
E_2 = — logarithm per cent. transmission of unknown.
C_1 = concentration of standard (here called " S ").
C_2 = concentration of unknown (as multiple or fraction of " S ").

Preparation of Suspensions of C. diphtheriæ.

The cultures after removal from the incubator were treated either with toluol or formalin; about 12 hours after the addition of antiseptic approximately 30 gm. of small glass beads were added and the cotton-wool stopper replaced by a rubber one. The bottles were then shaken vigorously for 10 minutes; in some cases it was found necessary to add one drop of caprylic alcohol to reduce the amount of foam. By this procedure the original film of organisms was reduced to a fairly uniform state, and small samples could be withdrawn by means of a pipette provided the mixture was agitated slightly while taking samples. Usually the sample was 10 c.c., but on occasions as much as 60 c.c. was necessary. The sample was centrifuged in a round-bottom 9·0 cm. × 2·8 cm. tube of 40 c.c. capacity until the supernatant was clear. Usually 30 minutes at about 3000 r.p.m. were necessary. After removing the clear supernatant liquid the tube was fixed to a vertical spindle so that it could be rotated. (This apparatus was constructed from an old hand-centrifuge.) By means of a glass rod, with an enlarged end covered with a rubber teat, the organisms could be broken up to form a smooth cream. After about one minute's grinding in this way, three drops of 20 per cent. saponin were added and grinding continued for a few seconds. Distilled water was then added, and the contents of the tube were washed out into a volumetric flask of suitable size. In this method we only desire to separate individual organisms to give a smooth emulsion free from lumps, and no attempt was made to disintegrate individual organisms by the grinding process.

The final dilution was made to such a volume that the transmission of light was between 30 and 70 per cent.—preferably as near 50 per cent. as possible. The procedure is made clear by the following example:

After shaking the culture a 10 c.c. sample was removed, centrifuged, and emulsified. It was diluted to 100 c.c. and the transmission found to be 44·0 per cent. This by calculation gives a value of 1·18 × " S ", and since 10 c.c. of culture was diluted to 100 c.c. this value must be multiplied by 10 (" S " being always recorded on the basis of 10 c.c. of original culture). The value is therefore 11·8 " S ". Where a volume of culture greater than 10 c.c. was used, the result should be reduced to the basis of 10 c.c. The following figures show the results obtained by taking a number of samples from one culture.

Culture (*a*): Four samples gave 11·2, 11·4, 11·55 and 11·55 " S ".
Culture (*b*): Five samples gave 11·5, 12·0, 11·85, 11·85 and 12·0 " S ".

For six individual cultures harvested after 40 hours growth we obtained values of 6·75, 7·20, 6·78, 7·50, 6·60 and 6·58 " S ", giving an average of 6·90 " S ". In addition we have measured the nitrogen contents of several suspensions; the ratios were in very good agreement with those obtained by measurement with the photo-electric colorimeter as shown in Table I. Three

TABLE I.—*Showing the Degree of Correlation Between Two Methods of Estimating the Amount of Bacillary Growth.*

Galvanometer swing in mm.		Percentage transmission.	"S" value.	Nitrogen mgm. per 10 c.c. emulsion.	"S"/N.
Distilled water.	Emulsion.				
320	145	45·3	1·14	0·285	4·01
320	157	49·1	1·03	0·242	4·25
320	168	52·5	0·93	0·226	4·11

cultures were centrifuged, the bacilli well washed, and suspensions were then measured as described, and micro-Kjeldahl nitrogen estimations carried out on a series of 10 c.c. quantities. The figures shown for nitrogen are the mean of four or five determinations. Measurement of the nitrogen content is not a very satisfactory method, since 10 c.c. of a suspension of 1 " S " value contains only about 0·24 mgm. of bacillary nitrogen. These results are typical of a large number which were obtained while testing the method of estimation given in this paper.

The photo-electric colorimeter used in this work is similar to that described by Smart (1931), but several modifications have been introduced. Owing to fluctuations in the mains voltage we were unable to secure constant illumination from this source. The substitution of a 12-volt car lamp (36 watt) fed from a car type accumulator gave us a steady light, but had the disadvantage that the light diminished in intensity slowly as the accumulator discharged. This was overcome by connecting a Philip's charger, giving approximately a 3-ampere charging current, and running this the whole time the colorimeter was in use. By means of a small resistance introduced into the lamp circuit the charge and discharge currents were made equal, and this gave us a steady light source. Usually the lamp and charger were switched on 30 minutes before using the apparatus to allow the light to become quite steady.

For the light source described above we replaced the condenser as used by Smart by a planoconvex lens arranged to give a parallel beam of light which passed through a filter, the cell, and then fell on the cathode of the photo-cell.

In order to compensate for the loss of light a galvanometer of higher sensitivity was used. This was an Ayrton Mather type with a coil resistance of 6000 ohms; it gave a deflection of 1400 mm. per micro-ampere at a scale distance of 1 metre.

The voltage for the photo-cell was obtained from the A.C. mains by means of a Westinghouse rectifier and was about 150 volts. A neon lamp was placed across the output terminals of the rectifier to absorb voltage fluctuations.

For the work described in this paper we used a red glass filter which cut off all light below 5600 Å. This gave a sufficiently close approach to monochromatic light for our purpose, and although Beer's law did not hold over

wide differences in the concentration of the bacillary emulsion, it did so very closely over the range used for our work.

TABLE II.—*Showing the Effect on Toxin Production of Simultaneous Variation of the Concentration of Maltose and Change in the Ratio "Surface Area/Volume of Medium".*

Volume of medium and A/V ratio.	Percentage maltose concentration.			
	0·2.	0·3.	0·4	0·5.
50 c.c.	Lf 59·0	71·0	82·0	55·0
2·0	"S" 12·9	10·4	9·9	24·0
	Lf/"S" 47·0	68·0	82·0	23·0
100 c.c.	Lf 74·0	82·0	92·0	47·0
1·0	"S" 12·7	16·4	11·2	25·3
	Lf/"S" 58·0	49·0	82·0	18·0
150 c.c.	Lf 68·0	78·0	73·0	43·0
0·7	"S" 23·3	22·8	26·0	20·3
	Lf/"S" 29·0	35·0	28·0	21·0
200 c.c.	Lf 56·0	64·0	68·0	38·0
0·5	"S" 23·8	22·9	22·0	19·7
	Lf/"S" 23·0	27·8	30·8	19·0

In the second experiment the medium was prepared in a similar manner, but wider concentrations of maltose were studied. These were 0·4, 0·8, 1·6 and 3·2 per cent., together with a control containing no maltose. At a concentration of 3·2 per cent. maltose growth was at first very heavy, but after 48 hours all the films dropped and the cultures showed no evidence of refilming. The results for this concentration have not been included in this paper.

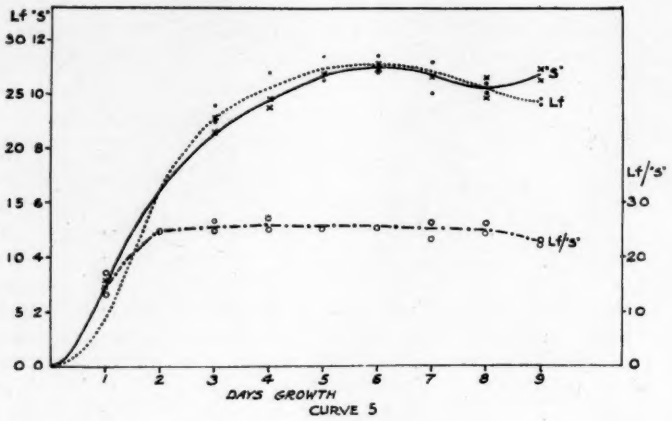
In this experiment the inoculation was made from a 24-hour "starter" culture which had been shaken to reduce it to as uniform a condition as possible. In this way we hoped to keep the size of the inoculum between reasonably narrow limits and thus secure some degree of uniformity from bottle to bottle.

The results of the second experiment are shown in Curves 5–9. Curve 5 shows the result obtained when medium containing no maltose was used, and it will be seen that the agreement of growth values between the pairs of culture filtrates harvested on any day was very close. After the sixth day there was a slight drop in growth values, followed later by a slight rise. This change had been found in a large number of experiments, and its frequent appearance suggests that it is not due to any error of technique. The ratio Lf/"S"* remains fairly constant from the second to the eighth day at a value of 25.

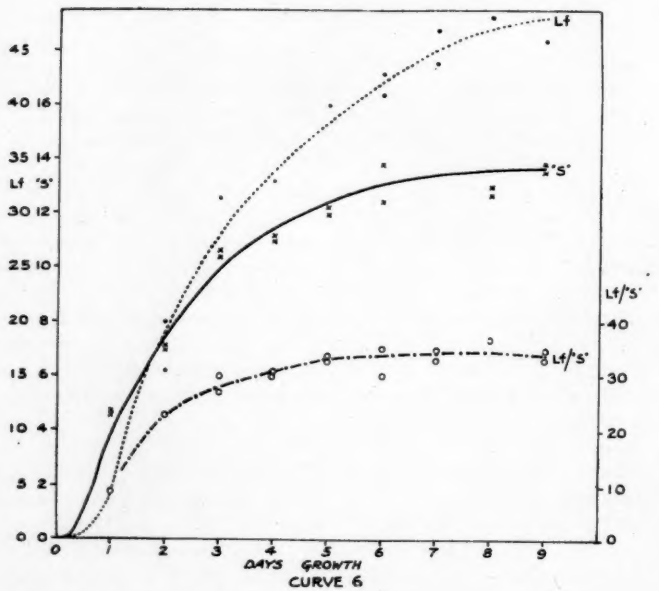
Medium containing 0·4 per cent. maltose gave the results shown in Curve 6. Here it will be seen that toxin production was greatly improved by this concentration of maltose, while the effect on growth was less pronounced. For an increase in growth of from 11 "S" to 14 "S" there had been an increase

* The ratio is given by $\frac{\text{Lf units per 10 c.c.}}{\text{"S"}}$, since "S" is based on 10 c.c. culture.

in the toxin value from 27 to nearly 50 Lf units per c.c. This change is reflected in the ratio Lf/"S", which rose throughout the experiment to a value of 35.

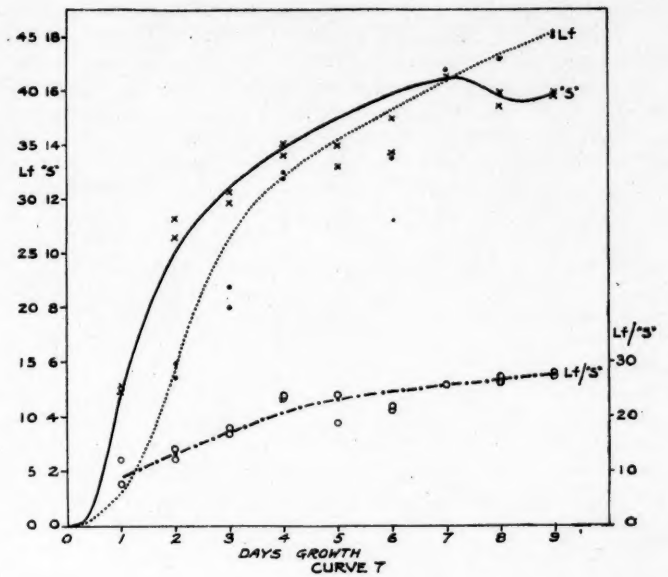


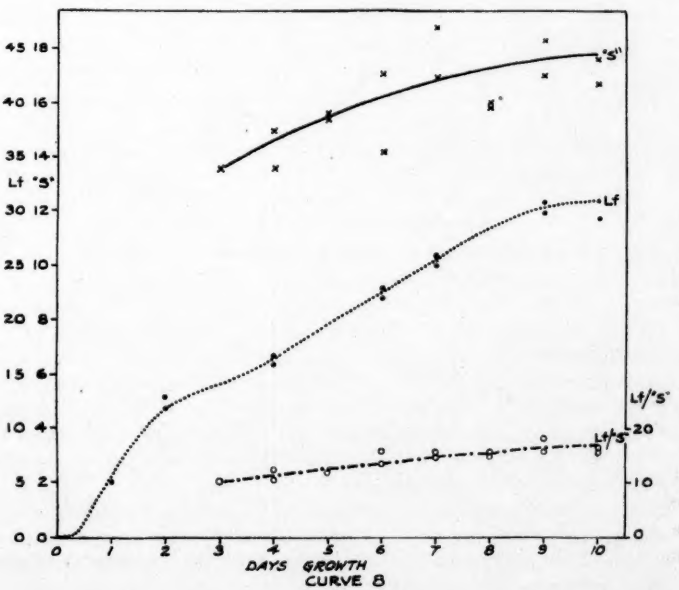
CURVE 5



CURVE 6

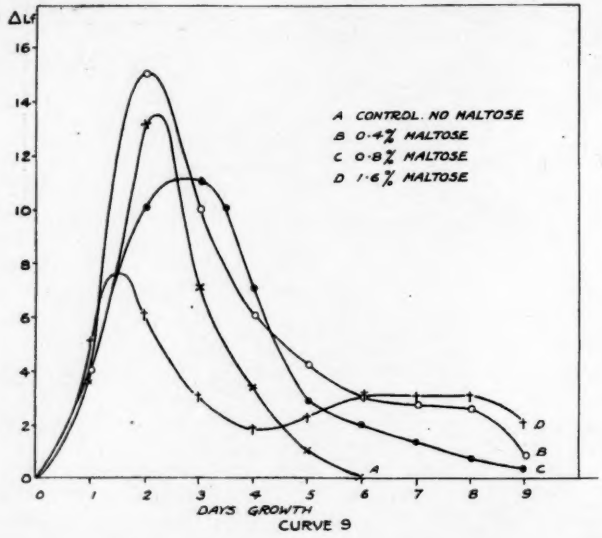
Increasing the concentration of maltose to 0·8 per cent. gave the results shown in Curve 7, from which it will be seen that there had been an increase in the amount of bacillary growth without a corresponding increase in the


Lf
"S"
Lf/"S"
Lf "S"
DAYS GROWTH
CURVE 7


"S"
Lf
Lf/"S"
Lf "S"
DAYS GROWTH
CURVE 8

amount of toxin produced. It will be seen, too, that agreement between pairs of cultures is less good than that obtained in the previous curves. In the first experiment it was found that the highest toxin values were obtained from medium containing 0·8 per cent. maltose, while in this experiment they were not so good ; it has been our experience that, while in some batches of medium this concentration of maltose may give good results, generally it is found to be rather too high, and better results are obtained with somewhat lower concentrations. The ratio Lf/" S " rose rapidly until the fourth day, and then more slowly, reaching a value of 27.

The effect produced by increasing the maltose concentration to 1·6 per cent. is shown in Curve 8. The irregularity of the growth values may be due to



CURVE 9

two causes. On this medium growth was at first very heavy, but within 48 hours there was complete collapse of the surface films, followed later by some signs of reformation. The irregular growth values may be influenced by the amount of new surface growth which took place in different cultures. On the other hand, the estimation of growth in these cultures was unavoidably delayed, and changes produced by standing during very hot weather may explain the irregular values found. As there was no contamination, we think the first explanation is probably the more correct one. The results indicate a very high growth value of 17–18 " S ", with a marked decrease in toxin production. The ratio Lf/" S " had a value of only 15.

In curve 9 we show the amount of toxin produced daily (ΔLf) plotted against the growth time, for media containing from 0–1·6 per cent. of maltose. These results show that while maltose in suitable concentrations does not appreciably increase the amount of toxin produced in a given time, it does

prolong the period over which toxin is produced, thus leading to higher final values in terms of Lf units per c.c. of culture filtrate. In curve D for media containing 1·6 per cent. maltose there is evidence of a second rise in toxin production, corresponding to the period when a new surface growth appeared.

The pH values of all cultures were measured colorimetrically at the time of harvesting. In the control medium the reaction dropped from an initial value of pH 8·0 to 7·4 on the first day, and then rose slowly to a final value of 8·8. In the presence of 0·4 per cent. maltose the pH fell slowly from the same initial value to 6·8 on the second day, after which it rose slowly to a final value of 8·6. At a maltose concentration of 0·8 per cent. the pH dropped to 7·0 at the first day, remained at this value until the fifth day, when it rose slowly to 8·1. After a drop to pH 6·8 at the first day cultures grown on medium containing 1·6 per cent. maltose remained within the limits 6·8–7·0 until the end of the experiment.

Attention has been drawn elsewhere (Pope and Llewellyn Smith, 1932) to the importance of the ratio "surface area to volume of medium" for high value toxin production. Since the round bottles which it has been customary to use in these laboratories limit the range of ratio obtainable, further experiments were carried out with square-section bottles, which allow the attainment of a constant surface area for varying volumes of medium. The results of a typical experiment under these conditions are given in Table II. The concentrations of maltose were from 0·2 to 0·5 per cent., and the ratio for "surface area/volume" increased from 0·5 to 2·0.

Each result given in the table is the mean value obtained from three culture filtrates, the results for individual culture filtrates in each group were in extremely close agreement both for "S" and Lf values. Thus at 0·4 per cent. maltose and A/V 1·0, the mean Lf value shown is 92 Lf units per c.c.; one filtrate appeared slightly stronger (95 Lf per c.c.), but this difference is almost within the limits of the flocculation method, since it means testing to an accuracy of 2 per cent.

The results from this experiment confirm our previous finding that maximal growth is not associated with maximal toxin production. For example, with 0·5 per cent. maltose and an A/V of 1·0 there was 25 × "S" growth for 48 Lf units giving an Lf/"S" value of 18, whereas at 0·4 per cent. maltose and the same A/V ratio, 11 × "S" growth gave 92 Lf units per c.c. with an Lf/"S" ratio of 82.

DISCUSSION.

The results presented in this paper show that maltose is of value for enhancing the production of toxin by *C. diphtheriæ* provided it is used in suitable concentration. Elsewhere (Pope and Llewellyn Smith, 1932) it has been suggested that maltose is of great value as a reserve energy source for the growing organism, since, while it is not directly used, its slow conversion into glucose prevents the marked acidity which would occur if an equivalent amount of glucose were added directly to the initial medium. There is evidence, however, that while maltose used in concentration as high as 1·6 per cent. does not give rise to marked acidity, the concentration of maltose used does influence

the reaction changes taking place during growth. These results are in agreement with those of Heller and Hazen (1932), who found that a concentration of maltose up to 3·0 per cent. did not produce an acidity comparable to that produced by much lower concentrations of glucose. Nevertheless our results again support the view advanced by one of us (Pope, 1932; and Pope and Llewellyn Smith, 1932) that the highest toxin production in this type of medium occurs when the final pH value is about 8·6, and it becomes important to select that concentration of maltose which allows suitable changes in the reaction during growth. The amount to be used will obviously depend on the nature of the medium and on the ratio of "surface area to volume of medium". In view of these facts we can only give here the optimal concentration of maltose for the particular type of medium used by us and under the same conditions of growth.

In the first series of experiments the highest toxin values were obtained from medium containing 0·8 per cent. of maltose, but later experimental work has shown this to be exceptional, and for most batches of medium prepared by tryptic digestion of beef muscle by the method described (Pope and Llewellyn Smith, 1932) the best results are obtained at a concentration of 0·4 per cent.

Inspection of Curves 5 to 8 shows that the amount of growth increased with increasing concentration of maltose without a parallel increase in the amount of toxin. In fact the toxin decreased at concentrations of maltose of 0·8 per cent. or more. This is clearly shown by the ratio Lf/"S", which reaches its highest value in medium containing 0·4 per cent. maltose. In the third experiment the results given in Table II again show a concentration of 0·4 per cent. maltose to give the best toxin production, particularly when the A/V ratio is adjusted to the optimal value. Since these results are typical of the effect of maltose in this type of medium, we feel that there is little advantage to be gained by the use of higher concentration of maltose than 0·4 per cent.

The improvements in the strength of toxin produced by *C. diphtheriæ* which have recently been reported from these Laboratories have been due to the provision of a suitable nitrogenous basal medium, together with the use of certain energy sources (sodium acetate, maltose, and in certain cases sodium lactate), and the determination of optimal conditions for toxin production. Sodium acetate was first used to enhance toxin production at these Laboratories in 1926, and its use reported in 1931 (Pope, 1931, 1932) and Pope and Llewellyn Smith (1932). Recently its use for enhancing toxin production has been advocated by Ramon and Berthelot (1932).

More recently Ramon (1933) has reported the use of both sodium acetate and glucose, by which means he has obtained culture filtrates containing 40 Lf units per c.c. Regarding the use of maltose he says: "L'emploi de sucres autres que le glucose, tels que maltose, saccharose, lactose, etc. . . ." "miel, etc. . . ., nous a donne des resultats le plus souvent inferieurs"—a finding which is contradictory to those obtained by Hazen and Heller (1932), Pope (1932), Pope and Llewellyn Smith (1932), and the results presented in this paper, where we are able to record toxin values far higher than any previously reported.

In this paper we do not wish to express any views as to the mechanism of toxin production, as we feel that it would be quite unjustified at this stage.

SUMMARY.

1. The influence of varying concentrations of maltose on the growth and toxin production of *C. diphtheriæ* has been studied.

2. A method for estimating the amount of bacillary growth is given.

3. The ratio Lf/" S " (toxin produced/growth produced) is used as an index for optimal conditions for toxin production.

4. A concentration of 0·4 per cent. maltose gives the best toxin results for the type of medium and conditions of growth used in this work. Toxin containing 92 Lf units per c.c. has been obtained by this method.

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SURFACE GROWTH AND TOXIN PRODUCTION OF *C. DIPHTHERIÆ*.

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IT is well known that in order to prepare diphtheria toxin of high value good surface growth is essential. Nevertheless we have found no record in the literature of the magnitude of the effect on toxin production which may be produced by variations in surface growth. Andrewes *et al.* (1923) state that " many workers believe that toxin is only produced when the bacteria form a pellicle. In this field our knowledge is most unsatisfactory ".

In the present paper we shall show that—

(1) Our strain of *C. diphtheriæ* (P.W. 8) can grow freely only at the air/liquid surface of the medium.

(2) That no growth can occur on the surface of the medium if this is occupied by a rigid film.

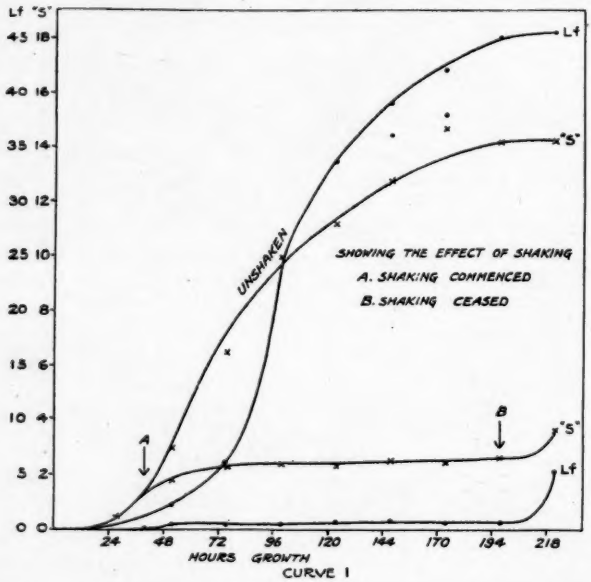
(3) Curves for the daily increase in growth and toxin production show that toxin production is closely associated with growth.

(4) Failure of growth entails cessation of further toxin production.

(5) The presence of toxin in a medium has no effect on the subsequent growth and toxin production of *C. diphtheriæ*.

EXPERIMENTAL.

Experimental proof of the first statement was obtained as follows : Medium prepared as previously described (Pope and Llewellyn Smith, 1932) was distributed into litre bottles in 200 c.c. volumes. Two groups of culture bottles were employed in the following manner ; each culture bottle was sown with a



CURVE 1

loopful of emulsified 24-hour broth culture (see Pope and Healey, 1933), and one group of cultures was allowed to grow undisturbed, while the other was gently shaken three times daily to prevent pellicle formation. The amount of bacterial growth was estimated by the method previously described (Pope and Healey, 1933). After this determination had been made the remaining portion of each culture was filtered, and the amount of toxin present determined by the flocculation method. In the group of culture bottles which was subjected to shaking, growth was allowed to proceed in a normal manner until it was judged that there was sufficient bacterial growth present to make accurate readings possible, and to control the fact that the initial growth-rate in the two groups was identical. The results of this experiment are presented in Curve 1, from which it will be seen that although the initial growth-rates were the same over the period during which both groups were undisturbed, they diverged greatly once shaking was commenced. Whereas the undisturbed section grew

normally and produced toxin, the cultures which were shaken showed very little increase in growth and a corresponding lack of toxin production. This condition persisted while the cultures were frequently disturbed, but at the end of the curve there was an increase in both growth and toxin, because over the week-end it was impossible to maintain the frequent disturbance of the cultures. As a result a pellicle formed ; growth occurred, and with it toxin production.

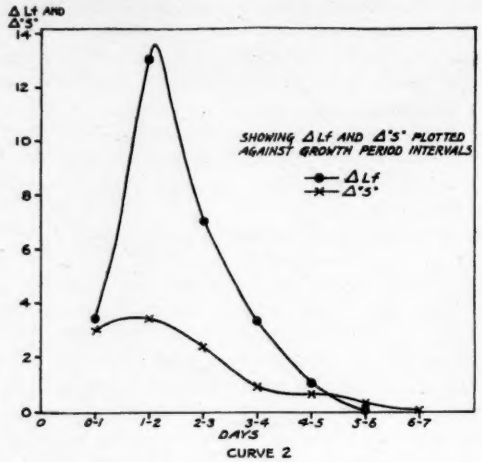
Evidence for the second point is provided by the following experiment. A batch of infusion-free medium (Pope, 1932) consisting of Difco proteose peptone, maltose, and inorganic salts (amongst which was calcium chloride), had been prepared and filled out into 200 c.c. quantities in litre bottles. These had been placed in a horizontal position in the incubator, but inoculation was delayed for some days. During this time a rigid film formed on the surface, possibly of calcium carbonate. The culture bottles were inoculated in the usual manner, the rigid film being broken only at the point where the needle entered the medium. After 24 hours, surface growth of the organism had taken place only where the rigid film had been broken. No definite growth had occurred in the depth of the medium, nor had the organism overgrown the rigid film, covering the remainder of the surface. This condition persisted unchanged for several days. The cultures were then gently shaken to destroy the rigid film, and in 24 hours the surface of the medium was completely covered by a film of *C. diphtheriæ*. The amount of toxin produced 7 days after shaking was normal for this type of medium.

Curves 2 and 3 provide the evidence for the third and fourth points. In the first of these we show Δ Lf (the daily increase in the amount of toxin present in the culture filtrates) and Δ " S " (the daily increase in the amount of bacilli present in the culture filtrates) plotted against growth intervals. From Curve 2 it will be seen that toxin production was maximal in the first- to second-day interval ; increase in bacillary growth was also maximal at this period. These two curves of Δ " S " and Δ Lf show the close relationship between growth and toxin production. In this experiment the final culture filtrates had a mean Lf value of 27 units per c.c. The medium used contained no maltose.

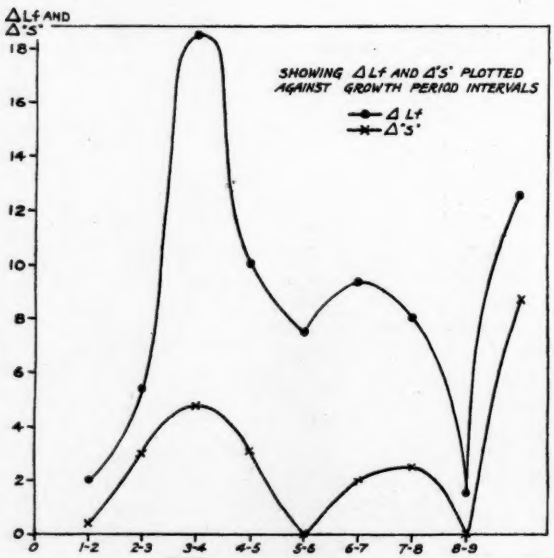
The third curve shows this relationship even more clearly. For this experiment medium containing maltose and other energy sources was used. Pellicle formation was both rapid and heavy, but at about the end of the second day's growth almost complete collapse of the film occurred. Vigorous new surface growth occurred, only to be followed later by a similar fairly complete collapse. Several culture bottles were removed daily, the Lf and growth values of which agreed closely. The values of Δ " S " and Δ Lf plotted as for Curve 2 show very clearly the effect of these successive refilmings on toxin production. In this medium the mean Lf value after 10 days' growth was 79 units per c.c. The parallelism between Δ " S " and Δ Lf in Curves 2 and 3 shows how intimately toxin production is bound up with the active surface growth of *C. diphtheriæ*.

The final point is covered by an experiment to find the effect of toxin already present in a culture medium on both growth and toxin production in that medium. Approximately 30 litres of toluolized culture filtrate was adjusted to pH 4·8, and the precipitated toxin removed by means of a Sharples

centrifuge. The precipitate was washed with a dilute buffer solution at pH 4·8 and added to a bulk of semi-synthetic medium (Pope, 1932), in which it dissolved



CURVE 2



CURVE 3

at pH 8·0. The medium, now containing toxin, was sterilized by filtration through Berkefeld candles, being distributed in 200-c.c. quantities in litre

bottles. A portion of the semi-synthetic medium without toxin was similarly treated to act as control. Several bottles of the " toxin-containing " medium were tested for their toxin content by the flocculation method. They were found to contain 20 Lf units per c.c.

The two sets of culture bottles were incubated and inoculated with *C. diphtheriæ* (P.W. 8), with the exception of a few bottles of the " toxin-containing " medium, which remained uninoculated to check possible deterioration of toxin during a 7-days' exposure to a temperature of 34° C. No such destruction of toxin occurred. After 7 days' growth, the amount of toxin in the culture filtrates of the two groups was compared. It was found that in the control group the culture filtrates had a mean Lf value of 30 units per c.c., the values of individual filtrates being very close to the mean value. For the " toxin-containing " medium the mean value was 50 Lf units. Thus since the controls gave a value of 30 units and this medium contained initially 20 units, there was no evidence that toxin had been destroyed or utilized by the growing organisms, or that the initial presence of toxin had in any way interfered with subsequent toxin production. The amount of bacillary growth produced also appeared to be uninfluenced by the initial presence of toxin in the medium.

DISCUSSION.

The results given in this paper show the importance of surface growth of *C. diphtheriæ* in connection with toxin production. In addition they also show the very close association between the actual growth of the organism and its toxin production.

A consideration of these results brings into prominence an important point. Having shown that free growth can only occur at the air/liquid surface, it becomes important to see how this affects the usual methods of producing toxin. Generally the bottles or culture flasks remain undisturbed throughout the incubation period, and on certain types of medium the film remains intact. If this occurs, then the film of organisms may be considered as occupying the only space where fresh growth might occur, and this in itself must limit further toxin production, since we have shown toxin production to be an activity of the growing organism. This would result in a limited toxin production of the type shown in Curve 2. It would therefore appear to be a logical proceeding to disturb the film when Lf determinations show that toxin production has almost ceased. Unfortunately, however, certain types of medium appear to be exhausted after the first surface growth has occurred, for little new pellicle formation occurs after shaking. On the other hand, certain types of medium show the spontaneous film regeneration of the type shown in Curve 3. For this type of medium there is obviously no necessity to disturb the film, since it breaks and re-forms naturally. This difference is in part due to known modifications in the composition of the medium, and partly also to unknown causes. We have been unable to detect any difference in surface tension between batches of medium which re-filmed spontaneously and those which did not when examined by the method described by Gaddum (1931). The time curves appeared identical within the limits of experimental error. Where the medium contains suitable energy sources, and does not re-film spontaneously there

appears to be some advantage to be gained by disturbance of the film at suitable periods. These must of course be determined for the medium in use.

We have previously pointed out (Pope and Healey, 1933) that on any given batch of medium, the largest amount of bacillary growth does not necessarily give the greatest toxin production, since the relationship between growth and toxin production is so much influenced by the cultural conditions (*e. g.* the ratio of surface area to the volume of medium and the composition of the medium). Nevertheless it is equally true that any artificial restriction in the amount of bacillary growth will restrict toxin production, and this explains why, in certain cases, toxin production is increased by the shaking which we have suggested.

That there can be no objection to this procedure on the grounds of possible destruction of toxin is shown by the fact that the initial presence of toxin is without effect on subsequent toxin production. Whatever the nature of toxin and the reason why the organism produces it, it would appear that it neither enhances nor hinders the activities of the organisms when present in the medium.

SUMMARY.

1. It has been shown that growth occurs freely only at the air/liquid surface.

2. That continual disturbance of the medium by preventing pellicle formation inhibits growth and toxin production. When the disturbance ceases film growth occurs, and with it toxin production.

3. Curves are given showing the close relationship between growth and toxin production.

4. It has been shown that the initial presence of toxin in a medium is without effect on the subsequent growth and toxin production of *C. diphtheriæ*.

5. A method for increasing the total toxin production is suggested.

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IMMUNOLOGICAL STUDIES WITH PHAGE-COATED BACTERIA.

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In the course of work on the classification of coli-dysentery phages (Burnet, 1933), it became clear that two phages might be serologically almost identical, yet show lytic activity against distinct bacterial species. Conversely, two phages of the same "resistance group" and with a similar range of lytic activity might be serologically unrelated. Facts of this sort suggested the working hypothesis that the phage particle was of a bipolar nature, one aspect being specifically related to the bacterial surface, the other toward the appropriate antiphage, and that these two aspects might vary independently. As a corollary it should follow that bacteria on which large amounts of phage had been adsorbed would be capable of reacting with the corresponding antiphage serum from which the normal bacterial antibodies had been removed. Such reaction could be detected experimentally either by absorption of antiphage or by direct agglutination of the phage-coated bacteria. Although further work has rendered it almost certain that this hypothesis of bipolar phage particles is incorrect in any literal spatial interpretation, the technique suggested has proved with some phages at least a useful method of studying certain aspects of the phage-antiphage reaction.

There are definite limitations to its use. Only phages which are readily adsorbed to the sensitive bacteria will give suitable emulsions, and a high titre antiphage serum is required. This has limited the work to two of the serological groups recently defined (Burnet, 1933), viz. the large group of small-plaque "resistance group II" phages, including phages C16 and D29, and the very large-plaque phages of resistance group I, C8 and C13.

Technique.

The following is the usual technique adopted for obtaining phage-coated bacteria. A suitable stock of freshly lysed culture is prepared and stored in the cold without filtration as soon as lysis is complete. The sensitive organism is washed off overnight agar plate cultures with broth and centrifuged. The packed bacterial sediment is then emulsified with nine times its volume of broth, giving a stock bacterial emulsion containing about 3×10^{10} bacilli per c.c. One c.c. of this emulsion is added to 9 c.c. of phage warmed to 48° C. in a water-bath left there for 20 minutes and then spun. The supernatant is discarded and replaced by another 10 c.c. of phage, the deposit emulsified and again exposed to 48° for 20 minutes before being spun. The process may be repeated several times, but for antiphage absorption experiments the fixation of the phage from 30 c.c. of lysed culture on 0·1 c.c. of bacterial sediment is sufficient. For agglutination experiments smaller amounts of bacteria for the same volume of phage should be employed.

After the final centrifugation the bacteria are emulsified in broth and 0·25 per cent. of formalin added. Heating at 48° for another 20 minutes suffices to kill the bacteria and inactivate any

free phage present. After a further centrifugation the sediment is resuspended in 0·2 per cent. saline to give an approximate concentration of 3×10^{10} bacilli per c.c. Owing to loss during centrifugation, and with some phages to unavoidable lysis during the process, the volume necessary will usually be somewhat less than the original 1 c.c. of bacterial emulsion. If the coated bacteria are to be used for agglutination experiments only, weak saline containing 0·25 per cent. formalin is used as diluent.

1. CHANGES IN THE SUSPENSION STABILITY OF PHAGE-COATED BACTERIA.

Bacteria heavily coated with phage C16 form unstable suspensions which sediment slowly even in 0·1 per cent. saline. This holds irrespective of whether the coated strain was S or R. Another manifestation of instability is a slow "muddy" agglutination by normal rabbit serum up to a serum dilution of about 1 : 320. There is some evidence that this instability is not so much due to the actual phage particles on the surface as to the changes induced in the bacterial surface by their specific activity, but the subject has not been adequately investigated. As long as the existence of these two forms of instability is borne in mind there is no difficulty in interpreting the results of agglutination experiments, the specific agglutination by antiphage sera being a much more rapid and frankly granular type of aggregation.

2. AGGLUTINATION BY ANTIPHAGE SERUM.

Most of these experiments have been carried out with phage C16 and its homologous antiserum. This phage is adsorbed to the sensitive strains, a smooth Flexner Y culture (YS) and a rough coliform (KR) in unusually active fashion. In one experiment, for example, 3×10^7 bacteria (KR) per c.c. absorbed almost exactly 90 per cent. of the phage particles from undiluted phage of titre 7×10^9, *i. e.* about 200 particles were absorbed by each bacillus. The antiserum available had a titre as shown in Table III of approximately 70,000—an exceptionally high value for an antiphage serum.

With these reagents agglutination of phage-coated bacteria is very easily demonstrable. It suffices to mix phage and bacteria in such proportions that from 20–200 phage particles are present for each bacillus, expose to a temperature of 48° for a few minutes and centrifuge. The deposit is emulsified to a suitable opacity in weak formolized saline (0·1 per cent.), and used as an agglutinating emulsion in the ordinary macroscopic agglutination technique. The antiphage serum naturally contains agglutinins, which are removed by absorption with the appropriate normal bacterium before carrying out the agglutination. Table I gives a typical protocol showing agglutination observed with normal and phage-coated YS against a standard Y agglutinating serum, and against the homologous antiphage serum absorbed with excess of normal Y bacteria.

TABLE I.—*Agglutination of Bacteria Coated with Phage C16.*

Serum.	Agglutinating emulsion.	Serum dilutions. 100.	200.	400.	800.	1600.	Control.
Normal agglutinating serum Y	Normal Y	+++	+++	+++	+++	+	−
	Phage-coated Y	++	+	±	−	−	−
Antiphage serum absorbed Y	Normal Y	−	−	−	−	−	−
	Phage-coated Y	+++	++±	++	+	−	−

Readings taken after 2 hours at 48°.

TABLE II.—*Agglutination of Phage-coated Bacteria.*

Sera.	*B. coli* R coated C16.				*B. coli* R coated C13.				Normal *B. coli* R.				Time of reading.
	40.	80.	160.	320.	40.	80.	160.	320.	40.	80.	160.	320.	
Normal rabbit serum	±	±	±	tr.	−	−	−	−	−	−	−	−	2 hrs.
	++	++	++	+	−	−	−	−	−	−	−	−	6 ,,
Anti C16 serum	+++	+++	+++	++	−	−	−	−	−	−	−	−	2 ,,
Absorbed coli R.	+++	+++	+++	+++	−	−	−	−	−	−	−	−	6 ,,
Anti C13 serum	±	±	tr.	tr.	−	−	tr.	−	−	−	−	−	2 ,,
Absorbed coli R.	++	++	++	++	+	+	++	+	−	−	−	−	6 ,,
Controls in 0·1% saline		−				−				−			2 ,,
		+				−				−			6 ,,

All dilutions were made in 0·1 per cent. saline.

TABLE III.—*Reciprocal Antiphage Absorption by Phages C16 and D29.*

Tested against phage.	Serum anti-C16.			Serum anti-D29.		
	Unabsorbed.	Absorbed C16.	Absorbed D29.	Unabsorbed.	Absorbed C16.	Absorbed D29.
C16	4·84	2·68	4·80	3·08	2·50	2·38
	(69,000)	(480)	(63,000)	(1200)	(320)	(240)
D29	4·32	2·80	2·65	3·85	3·78	2·60
	(21,000)	(630)	(450)	(7100)	(6000)	(400)

The figures shown are the logarithms of serum dilutions (read off from curves similar to those in Fig. 1) at which 20 per cent. of the plaques in the control would be present.

The agglutination is fairly rapid, and closely resembles an O type granular agglutination. The floccules, however, are slightly looser in texture than those produced by normal agglutination of a smooth dysentery bacillus.

Agglutination is specific for the phage, and independent of the type of organism on which it is absorbed. The antigenically dissimilar strains KR and YS are both agglutinable by the specific antiphage when coated with C16, but not by the antiserum to the large plaque phage C13. Similarly when KR is coated with phage C13, it is agglutinated by the corresponding antiphage serum but not by the C16 antiserum (Table II).

The agglutinability results from the absorption of phage particles on the bacterial surface, and is not due to soluble substances present in the lysed culture. An active filtrate of C16 was filtered through a membrane of average pore size 60$\mu\mu$ (Elford) capable of retaining the phage completely. This ultra-filtrate had no influence on the agglutinability of YS. The same stock phage was washed on a similar membrane by successively reducing its volume to one-tenth and making up to the original with broth before continuing the filtration. By repeating this process five times the soluble bacterial products can be reduced to 1/100,000 of their original concentration with only slight loss of bacteriophage activity. Such a purified preparation induced the characteristic agglutinability by antiphage to the same degree as a crude filtrate adjusted to contain the same number of lytic particles.

Finally it has been shown that sera from which the greater part of the antiphage has been absorbed by coated bacteria have concomitantly lost their power to agglutinate such bacteria.

3. ABSORPTION OF ANTIPHAGE: RECIPROCAL ABSORPTION OF TWO RELATED ANTIPHAGE SERA.

As I have described elsewhere (Burnet, 1933), there is a large group of small-plaque Group II phages, all of which are inactivated by antisera made against two typical examples, C16 and D29. No two of these phages, however, seemed to be completely identical antigenically, a given serum always neutralizing the homologous phage to a higher titre than any other. The differences between C16 and D29 in this respect are illustrated by two curves in the paper referred

to. It was therefore of considerable interest to carry out a complete cross-absorption experiment with these phages and their antisera.

Technique.

Bacteria (*B. coli* R.) were coated with phage C16 or D29, and made up to standard concentration in broth according to the method described. One c.c. of bacterial emulsion was added to 4 c.c. of antiphage serum diluted 1 : 80, giving a final serum dilution of 1 : 100, and the mixtures kept at 48° for 20 minutes with occasional shaking and then left in the refrigerator overnight. Next morning they were heated again to 48° for a few minutes and then centrifuged. The supernatant liquids were removed and serial dilutions prepared. Unabsorbed serum diluted 1 : 100 was exposed to the same manipulations, many previous experiments having shown that antiphage sera absorbed with *normal* bacteria showed no alteration whatever in their antiphage titres.

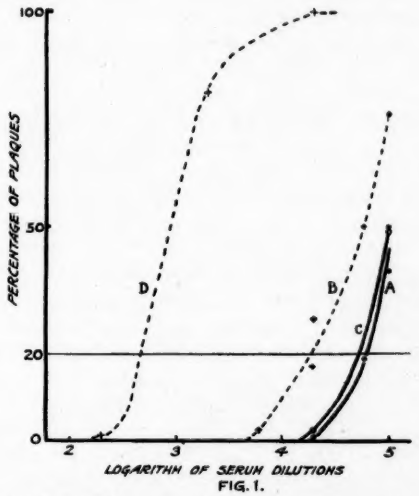

FIG. 1.—To show method of plotting results of neutralization experiments to obtain antiphage titre. Serum anti-C16 unabsorbed and absorbed with D29, tested against both phages.

A = Serum anti-C16 unabsorbed v. phage C16.
B = „ „ „ „ D29.
C = „ „ absorbed with D29 against C16.
D = „ „ „ „ „ D29.

Serum dilutions were now added to an equal volume of phage diluted in broth, so that the final plating of 0·02 c.c. should show approximately 100 plaques. The tubes were then placed in a water-bath at 48° for 1 hour, and left a further hour at room temperature. The phage content was then estimated by plating in duplicate 0·02 c.c. of each mixture on plates spread with a young culture of the sensitive strain. Plaque-counts made next morning were plotted as percentages of the control against logarithms of serum concentrations, and the point at which the curve crossed the 20 per cent. level taken as the serum titre against the phage in question.

The results with phages C16 and D29 and their respective antisera are summarized in Table III. Fig. 1 shows the detailed results of antiphage

titrations of serum anti-C16 unabsorbed and absorbed with D29, against both phages.

Although it was not possible to exhaust the sera completely, it is perfectly clear that the same type of reciprocal absorption relation holds between two serologically related phages as between related bacterial species or chemically similar artificial antigens (Avery, Goebel and Babers, 1932). Absorption with the homologous antigen lowers the titre uniformly for homologous and heterologous; absorption with heterologous antigen lowers the titre against itself, but leaves the activity against the homologous antigen almost unaffected.

4. ADSORPTION OF FORMALIN-KILLED PHAGE TO BACTERIA.

The technique described makes it possible to determine whether phage inactivated by formalin is still capable of becoming attached to bacteria sensitive to its action. Using phage C16 inactivated with 0·25 per cent. formalin, clear evidence was obtained, both by agglutination with suitably absorbed antiphage serum and by the absorption of antiphage technique, that the formalin-inactivated phage could be adsorbed in antigenically reactive form on the surface of suitable bacteria. The agglutination was not quite so rapid as in a corresponding preparation made with living phage but was quite unequivocal. An absorption of antiphage experiment was set up in which formalin-killed bacteria, subsequently washed and left in contact with active phage, were compared with initially living bacteria exposed to formalin-inactivated phage. The final suspensions diminished the antiphage titre of the serum used to almost identical levels. The drop in titre was of the same degree as would have been expected with similar amounts of bacteria coated with phage according to the usual technique.

DISCUSSION.

The experiments described above are all consistent with the obvious interpretation that phage fixed on the surface of bacteria can combine with antiphage, and that such antigen-antibody union results in the formation of a film of denatured globulin over the bacillus, and consequent agglutination in the presence of salts.

An alternative explanation might be sought along lines suggested by the work of Bruce White (1933) on the more deeply placed antigenic constituents of the Salmonellas. If the action of phage on resting bacteria is to expose in some fashion an antigen not normally present on the surface, and if this component is present in an antigenically effective form in the lysed cultures used for immunization, an apparent specific agglutination might occur. This type of explanation has been carefully considered, and it seems probable that there *are* definite physical and perhaps antigenic changes in the bacterial surface on which phage has been absorbed even in the case of formalin-killed bacteria. A number of writers from d'Herelle onwards have noted that normally stable bacteria may agglutinate under the influence of certain phages quite apart from the presence of serum. This agglutination may occur even with dead

bacteria (Bordet and Renaux, 1932). Brill (1932) has recently described a phenomenon, "serophago-agglutination", which is of interest in relation to the present work. He used a coliform strain from which three variants had been obtained—S agglutinable, S inagglutinable and R. The inagglutinable strain could be rendered normally agglutinable by the homologous serum if a drop of an active phage were added to each tube of the agglutination set up. Heat-killed (60°) bacteria could be rendered similarly agglutinable. No experiments with antiphage sera were reported. Brill's results bear out the suggestion, which has arisen several times in the course of the present work, that when certain phages are adsorbed to the surface of resting or even dead bacteria, definite changes in the bacterial surface are induced in addition to those resulting from the presence of a new (phage) surface antigen. In general these changes tend to diminish the suspension stability of the bacteria.

The increased instability may play some part in rendering the specific agglutination by antiphage more rapid and complete than it would otherwise be, but there is no evidence that any corresponding abnormal bacterial antibody is present in the antiphage serum. This hypothetical antibody is certainly not present in antisera produced by immunization with normal S or R dysentery bacilli, nor in antiphage sera made by immunization with other phages grown on the same bacterial strains. The specificity of the reaction shown in the comparison between phages C16 and C13, and the ability of the coated bacteria to absorb only the homologous antiphage, seem to make it certain that the simplest explanation is the correct one.

At the present time any discussion as to whether the phage-antiphage reaction resembles the toxin-antitoxin union or the aggregation reactions found with protein and bacterial antigens is rather futile. The present results, however, may serve the purpose of showing how the same antibody can function in the ordinary phage "neutralization" experiment as an antitoxin-like agent and with coated bacteria as an agglutinin. This is in harmony with the now almost universal view that serum antibodies of all sorts are essentially similar in their nature and function, and that the different manifestations of antigen-antibody union are entirely due to the different physical conditions in which the antigen may be present.

The fact that two related phages show the same sort of reciprocal absorption relationship as exists between, say, two races of Flexner dysentery bacilli or between the α and β-glucoside-containing antigens of Avery, Goebel and Babers (1932) is of some interest. Taken along with the last-mentioned example, it makes one wonder whether orthodox bacteriological reasoning is always correct in assigning a mosaic structure to bacterial antigens on the strength of this type of absorption relationship.

CONCLUSIONS.

Bacteria on which certain phages have been adsorbed can be specifically agglutinated by antiphage serum, and can be used to absorb antiphage from an immune serum.

Cross-absorption experiments by this technique have been carried out with two antigenically related phages and their antisera.

With one phage it has been shown that formalin-inactivated phage can be adsorbed by bacteria, and endow them with the specific reactivity toward the corresponding antiphage.

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A SPECIFIC SOLUBLE SUBSTANCE FROM BACTERIOPHAGES.

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IN his early writings d'Herelle (1921) laid considerable stress on the existence of a soluble enzyme, lysin, produced by bacteriophage. The evidence for this was rather slight, and in 1926 d'Herelle stated that there was probably no satisfactory proof of its existence. More recently Sertic (1929) has described a coli-phage, "Fcz", which produces relatively small plaques surrounded by a zone of partial clarification, which steadily enlarges on further incubation of the culture. This outer zone contains phage only in the immediate neighbourhood of the plaque proper. The more peripheral parts contain an agent capable of inducing the vitreous change in coli cultures when in sufficient concentration but not transmissible in series. Ultra-filtrates of lysed cultures free from phage particles were capable of showing the lytic effect, and when used for immunizing rabbits produced antisera capable of inactivating both the soluble "lysin" and the phage. Asheshov (1925) also found that the phage-free ultra-filtrate of a large plaque phage could induce the appearance of antibodies capable of inactivating the phage in question.

Whether these findings of Sertic have any general significance is not yet clear. The phage Fcz is clearly an unusual type. The large plaque phages with a broad semi-vitreous edge which I have tested along similar lines all showed phage up to or a little beyond the region showing any alteration whatever in the culture. If it could be shown that a *single* antibody was produced by the injection of ultra-filtrates of Fcz, capable of inhibiting both the "lysin" and the phage itself, this would have an important bearing on the nature of the phage-antiphage reaction. From Sertic's data it seems equally possible that two independent antibodies were present in his serum, and that the

appearance of a soluble lysin may be characteristic only of certain rare phage-bacterium interactions.

In the course of work on some aspects of the phage-antiphage reaction, it was found that an ultra-filtrate of phage C16 was capable of " blocking " the homologous phage-antiphage reaction to a significant extent. Since no reaction of this type seems to be described in the literature, this blocking effect has been studied in the hope that it might throw some light on the mechanism of the phage-antiphage reaction. As will be shown, the agent in the ultra-filtrate responsible for the blocking is specific for the phage, and throughout the paper the non-committal term "specific soluble substance" (S.S.S.) has been used.

Technique.

The ultra-filtrates were prepared for me by Dr. W. J. Elford from freshly lysed cultures unless otherwise stated. After a preliminary filtration through a Seitz disc or a fairly permeable membrane, they were passed through a membrane of suitable permeability to hold back the phage particles completely. The filtrates were tested for absence of phage by direct plating on agar, and by the addition of 1 c.c. to a young broth culture of the sensitive strain. They invariably proved free of phage.

In order to demonstrate the existence of the specific soluble substance, the most convenient technique is to make successive dilutions of antiserum in ultra-filtrate as well as in broth, leave these dilutions for an hour or two at room temperature and in the refrigerator overnight, and titrate against a standard dilution of phage in the usual way. A more accurate estimate of the activity of an ultra-filtrate can be obtained, once its approximate activity is known, by choosing an optimal mixture of antiserum and ultra-filtrate, which is left overnight in the refrigerator, and further dilutions in broth made just before titration.

The following protocol will illustrate the methods. The experiment was designed to estimate the relative amount of specific soluble substance in two ultra-filtrates of phage C16, (A) filtered immediately lysis was complete, and (B) allowed to remain 7 days at 37° in contact with semi-resistant bacteria.

Experiment 1.—To 6 sets of 3 sterile tubes 0·45 c.c. quantities of the following were added, (1) broth, (2) ultra-filtrate A, (3) ultra-filtrate B, (4, 5 and 6) ultra-filtrate B diluted with broth 1:2, 1:4 and 1:8 respectively. A 1:100 dilution of antiserum C16 was made in broth, and from this successive 10-fold dilutions were made in each set of tubes by successive transfer of 0·05 c.c. quantities. The mixtures were shaken and left for 4 hours at room temperature, then placed in the refrigerator overnight. The following morning a second series of dilutions in broth was made from the first tube of each set, *i. e.* the one containing 1 : 1000 dilution of serum. Each set now consisted of 5 tubes, 3 containing 1 : 1000, 1 : 10,000 and 1 : 100,000 serum dilutions in the fluid being tested, the other being 10- and 100-fold dilutions in broth of the first tube corresponding to 1 : 10,000 and 1 : 100,000 dilutions of serum. 0·05 c.c. from each tube was now mixed with 0·05 c.c. of a standard dilution of C16 phage in broth calculated to give between 50 and 100 plaques on final plating. The tubes were shaken, placed in a water-bath at 48° C. for 1 hour, left a further hour at room temperature, and 0·02 c.c. from each plated in duplicate on the sensitive bacterial strain. Table I shows the counts obtained. It will be seen that the blocking effect is easily demonstrable and can be estimated quantitatively, the ultra-filtrate from the week-old culture being approximately twice as active as that from the recently lysed culture.

THE SPECIFICITY OF THE " BLOCKING " EFFECT.

The " blocking " effect on antiphage action is shown to a variable degree by all the ultra-filtrates of phage C16 which have been examined. The

phenomenon seemed at first capable of three explanations: (*a*) It was a non-specific inhibition of the phage-antiphage union by some product of the disintegrated bacteria; (*b*) the soluble substance was the lysin originally assumed by d'Herelle to be produced by the phage particles; or (*c*) the soluble substance was the specific antigen of the bacteriophage surface present in soluble form either as haptene or complete antigen. On the first hypothesis the effect should be nonspecific, blocking any phage-antiphage reaction, while if either (*b*) or (*c*) is correct the blocking will be specific for the phage involved. This alternative was susceptible to experimental test. Three phages, C16, D44 and L, all lysing the one strain Ys and serologically distinct, gave ultra-filtrates showing a blocking effect strictly specific for the homologous phage (Table II). The ultra-filtrate from another phage lysing this strain (D20) was almost inactive in this respect, but again none of the other ultra-filtrates inhibited the D20 phage-antiphage union. The soluble substance present therefore is specifically related to the phage from which it was obtained.

TABLE I.—*Titration of Phage C16 S.S.S. by Two Methods.*

I.

Serum dilution.	Fluid in which serum dilutions were made.					
	Broth.	Ultra-filtrate A.	Ultra-filtrate B.	B. 1 : 2.	B. 1 : 4.	B. 1 : 8.
2000	0	5	31, 22	5, 7	1	0
20,000	0	59, 77	82	73, 81	84, 70	29, 33
200,000	42, 48	80	90	95, 82	61, 106	94, 75
Nil	84, 81					

II.

Serum dilution.	Fluid in which 1 : 1000 serum dilutions were made.					
	Broth.	Ultra-filtrate A.	Ultra-filtrate B.	B. 1 : 2.	B. 1 : 4.	B. 1 : 8.
20,000	3, 0	34, 72	56, 74	56, 53	38, 28	6, 11
200,000	45, 35	95, 74	66, 86	78, 72	67, 77	63, 68
Nil	88, 80					

Figures represent plaque counts from 0·02 c.c. of mixure of standard phage dilution with serum dilutions shown.

THE RELATION OF THE S.S.S. TO D'HERELLE'S "LYSIN".

It has been shown that sensitive bacteria coated with phage are specifically agglutinable by corresponding antiphage sera, and can be used to absorb out the *phage-neutralizing* activity from the serum (Burnet, 1933). Further washed phage particles could be used to confer the specific agglutinability on the bacteria. These facts point strongly to the conclusion that for each phage there is a specific antibody produced, whose activity is manifested both in aggregation reactions, such as agglutination of coated bacteria, diminished filterability (Andrewes and Elford, 1933) or frank precipitation, and in the characteristic inhibition of lytic activity. The view that an antiphage serum acts by inhibiting the activity of a soluble lysin produced by the phage can hardly be reconciled with these experimental results. However, it was thought necessary to determine as definitely as possible whether the S.S.S. present did represent the lysin of d'Herelle's hypothesis. The ultra-filtrates showed no ability to clarify appropriate bacterial growths either in broth or on agar

surface. This might have been due to the small quantity present. A more definite result was obtained by absorption experiments with sensitive bacteria. Any lysin whose function was to hydrolyse the bacterial cell-wall would inevitably be adsorbed by an excess of the appropriate bacteria. Experiment 2 shows that such adsorption does not occur.

TABLE II.—*Specificity of S.S.S. Blocking Effect.*

A. *Action of Ultra-filtrates on the C16 Phage Antiphage Reaction.*

Serum dilution.	Fluid in which serum dilutions were made.				
	Broth.	C16 ultra-filtrate.	D20 ultra-filtrate.	D44 ultra-filtrate.	L ultra-filtrate
2000	0	0, 0	0	0	0
20,000	0, 3	48, 53	0, 0	2, 0	1, 1
200,000	37	65	54	36	37
Nil	71, 87				

B. *Action of Ultra-filtrates on L Phage-Antiphage Reaction.*

Serum dilution.	Fluid in which serum dilutions were made.			
	Broth.	L ultra-filtrate.	C16 ultra-filtrate.	D20 ultra-filtrate.
20	0	0	0	0
200	1, 1	2, 3	1, 0	1, 3
2000	25, 27	97, 94	33, 20	30, 25
20,000	120, 96	119, 103	97, 106	105
Nil	108, 126			

C. *Action of Ultra-filtrates on D44 Phage-Antiphage Reaction.*

Serum dilution.	Fluid in which serum dilutions were made.				
	Broth.	D44 ultra-filtrate.	C16 ultra-filtrate.	L ultra-filtrate.	D20 ultra-filtrate.
200	0	0	0	0	0
2000	0	6, 9	0	0	0
20,000	23, 15	15, 26	20, 12	..	..
Nil	38, 37				

Experiment 2.—The sensitive strain WR and the resistant WR/D4 were grown on agar, and the surface growth from three plates each was washed off with 10 c.c. of broth and spun. The deposit was in each case emulsified in a little broth to give a volume of 1 c.c. and added to 9 c.c. of C16 ultra-filtrate. The mixtures were kept at 48° for 30 minutes and then placed in the refrigerator till next day. They were then centrifuged, and the supernatant fluids rendered sterile by passage through gradocol membranes (A.P.S. 0·8 μ). The S.S.S. content of these fluids was then estimated by the technique described above, unabsorbed ultra-filtrate and broth being used at the same time as controls. The result in Table III shows that only an inappreciable amount of S.S.S. has been removed by these manipulations.

TABLE III.—*Failure of Bacteria to Absorb S.S.S.*

Serum dilution.	Fluid in which dilutions were made.			
	Broth.	Ultra-filtrate C16.	Ultra-filtrate C16, absorbed WR.	Ultra-filtrate C16, absorbed WR/D4.
2,000	0	3	1	0
20,000	0	83, 78	60, 89	64, 55
200,000	36, 33	95	60	80
Nil	75, 83			

If the S.S.S. does not represent a lysin, the only alternative is to regard it as equivalent to similar specific substances, antigens and haptenes, which are liberated in bacterial cultures, and which are antigenically identical with the structural units of the bacterial surface.

HEAT RESISTANCE OF S.S.S.

The blocking effect of an ultra-filtrate is completely destroyed by boiling for a few minutes. A series of experiments showed that inactivation by heat commenced about 65°, and was practically complete after 30 minutes at 90°. Table IV shows the percentages of plaques obtained when the standard form of experiment was set up with ultra-filtrate of C16 heated for 30 minutes at the temperatures shown.

TABLE IV.—*Heat Resistance of S.S.S.*

Serum dilution.	Temperature to which ultra-filtrate was heated for 30 minutes.								
	Unheated.	65°.	70°.	75°.	81°.	85°.	90°.	93°.	Broth control.
2000	2·8	0	0	0	0	0	0	0	0
20,000	95	74	58	35	16	10	2·1	0·7	0·9
200,000	100	100	100	100	100	76	62	50	66

It appears, therefore, that the rate of heat inactivation changes relatively slowly with rising temperature, which renders it unlikely that the underlying process is a heat denaturation of protein, and is not inconsistent with the possibility that the agent is a rather labile polysaccharide.

THE PRODUCTION OF S.S.S. FROM PHAGE.

Attempts to increase the amount of S.S.S. in the ultra-filtrate by various manipulations of phage-containing filtrates have been unsuccessful. Repeated freezing and thawing reduced the phage titre slightly, but there was no evident increase of S.S.S. in the ultra-filtrate. Alkalinization to pH 9·5 and re-neutralization after being kept for 30 minutes at 45° inactivated approximately two-thirds of the phage, but the ultra-filtrate was less active than that from the control phage. A definite increase, however, was observed with ultra-filtrate from a lysing culture that was allowed to remain 7 days at 37° in contact with semi-resistant growth (see Experiment 1). From most batches of phage C16 filtered as soon as lysis was complete very similar amounts of S.S.S. were demonstrable, but in this particular case approximately twice the standard amount was present.

REVERSAL OF THE PHAGE-ANTIPHAGE UNION BY EXCESS OF S.S.S.

The union of S.S.S. and antiphage proceeds slowly—as far as one can judge more slowly than the corresponding phage-antiphage union. It is still incomplete after 2 hours at 48° C. Any attempt to demonstrate reversal of the phage-antiphage union would therefore require a large excess of S.S.S. The following experiment shows that a certain degree of reversal can be obtained:

TABLE V.—*Reactivation of "Neutralized" Phage by S.S.S.*

Original phage concentration.	Secondary dilution in—		Equivalent plaques in absence of serum.	Final concentration of serum.	Time of plating after secondary dilution.			
					Immediately.	2 hrs.	4·3 hrs.	28 hrs.
10^{-2}	Broth	10^{-1}	$1{\cdot}3 \times 10^{5}$	1 : 25,000	*800*	165	32	0
		10^{-2}	$1{\cdot}3 \times 10^{4}$	1 : 250,000	128	76	40	1
	S.S.S.	10^{-1}	$1{\cdot}3 \times 10^{5}$	1 : 25,000	*1000*	*1000*	*800*	*800*
		10^{-2}	$1{\cdot}3 \times 10^{4}$	1 : 250,000	167	208	205	342
10^{-3}	Broth	10^{-1}	$1{\cdot}3 \times 10^{4}$	1 : 25,000	119	15	4·5	0
		10^{-2}	$1{\cdot}3 \times 10^{3}$	1 : 250,000	14	8	5	0
	S.S.S.	10^{-1}	$1{\cdot}3 \times 10^{4}$	1 : 25,000	135	110	113	128
		10^{-2}	$1{\cdot}3 \times 10^{3}$	1 : 250,000	12·5	21	22	42

Plaque counts in italics are estimates only ; the other figures are averages of two counts.

Experiment 3.—Phage C16 was diluted 1 : 100 and 1 : 1000 in broth, and two sets of 0·05 c.c. of each dilution added to 0·05 c.c. of antiserum diluted 1 : 2500. After one hour's contact at 37° the mixtures were diluted by adding either 0·4 c.c. of warmed broth or homologous ultra-filtrate. A further dilution of 1 : 10 was also made into the appropriate diluents. There were now four tubes corresponding to each original phage dilution, representing 1 : 5 and 1 : 50 dilutions of the original phage-serum mixtures in both broth and ultra-filtrate. As soon as the dilutions were completed 0·02 c.c. quantities were plated in duplicate from each tube on to the sensitive strain, and similar amounts were plated at subsequent intervals, the tubes being kept and all manipulations carried out in the hot room at 37°. All the tubes remained clear of growth for 24 hours, and any increase in plaques could only be due to reversal of phage-antiphage union. The results of this experiment are shown in Table V.

It will be seen that the undiluted S.S.S. preparation is capable of stopping the further action of 1 : 25,000 serum, but not of showing demonstrable reactivation of phage particles.

When the serum concentration is reduced to 1 : 250,000 the presence of the S.S.S. allows slight but definite increase in the number of phage particles capable of giving rise to plaques. With this phage there is no suggestion of any reactivation of phage by dilution of the phage-antiserum mixture in broth alone. These findings are in accordance with the results of Andrewes and Elford (1933), who found that reactivation by dilution occurred only with certain phages (C36, not C16), and to a very limited degree.

ANTIGENICITY OF S.S.S.

Two rabbits were inoculated intravenously with increasing amounts of the strongest ultra-filtrate of C16 (Preparation B of Experiment 1), a total of 7·5 c.c. being given over 3 weeks. They were bled a week after the last injection and tested for antiphage activity against C16. With the standard technique only barely significant reductions in plaque counts were obtained with undiluted serum, and the experiment was repeated, leaving the phage and serum in contact for a further 24 hours at room temperature. As controls the serum from each rabbit taken before the commencement of immunization and an agglutinating serum against Ys, the strain used in the preparation of the ultra-filtrate, were employed. Table VI shows that rabbit 66 developed a slight but quite distinct neutralizing power after immunization with ultra-filtrate; rabbit 65 was not in good condition, and perhaps for this reason failed to show any increase in the slight antiphage activity present before immunization. Both rabbits, however, produced agglutinins against Ys to a titre of 1 : 1000.

TABLE VI.—*Antiphage Activity of Sera Produced by Immunization with Ultra-filtrate of C16.*

Serum (diluted 1 : 2).	Percentage of plaques on control produced after—	
	1 hr. at 48°.	1 hr. at 48° + 24 hrs. R.T.
R65 (preliminary)	55	32
R65 (immunized)	66	27
R66 (preliminary)	92	79
R66 (immunized)	55	9·5
Anti Ys aggl. serum	100	83

When one considers that a similar course of injections of crude phage C16 gave antiphage titres from 5000–20,000, and that from Andrewes and Elford's (1933) data on the extreme difficulty of detecting absorption of antiphage by phage it is quite possible that there may be as much phage antigen in soluble form in the ultra-filtrate as is present in the phage particles of an ordinary filtrate, it is clear that the antigenicity of the S.S.S. is extremely low. This was also shown by using ultra-filtrate as a secondary stimulus. Injection of 3 c.c. into a rabbit previously immunized with C16 gave a just detectable increase in antiphage activity, while a similar injection of washed phage increased it approximately tenfold.

SPECIFIC PRECIPITATION.

All the phage-free ultra-filtrates which have been mentioned failed to give any visible precipitation when mixed with homologous antiserum with the exception of that from C16. This gave a slight clouding with an antibacterial serum against the organism (Ys) on which the phage was grown, and a more definite but still slight finely granular precipitate with anti C16 serum absorbed of its agglutinins with S and R races of Y. The reagents used were all clarified by membrane filtration and set up in a sterile condition. A very faint opalescence appeared after an hour in a water-bath at 48°, and fine granules were visible with a lens at 4 hours. After standing overnight at room temperature a slight deposit was present at the bottom of the tube. This absorbed serum had no such action on ultra-filtrates (or phage-containing filtrates) of D20, D44 and L, all grown on the same strain Ys.

COMMENT.

These experiments confirm the findings of Asheshov and Sertic that phage-specific soluble substances are present in lytic filtrates. In the case of C16 there seems to be no evidence that these represent a " lysin ". As far as can be judged from the present experiments, the phage-specific soluble substances are simply analogous to the similar bodies liberated by bacteria or viruses such as vaccinia (Craigie, 1932 ; Wilson Smith, 1932). They can best be regarded as fragments of the material which normally forms the specific surface antigen of the phage particle. It is possible that the S.S.S. is mainly in the form of a carbohydrate haptene, its slight antigenicity being accounted for by adsorption of the haptene to some of the various colloidal constituents of the ultra-filtrate, but no direct evidence is available on the point. Apart from establishing another point of similarity between phages and such a typical virus as vaccinia, the existence of phage S.S.S. seems to have no special significance, either in regard to the phage-antiphage reaction or to the interaction between phage and bacterium.

CONCLUSIONS.

1. A phage-specific soluble substance is present in phage-free ultra-filtrates of lysed cultures ; it can be demonstrated by its blocking effect on the specific phage-antiphage reaction.

2. The S.S.S. is not adsorbed by bacteria sensitive to the corresponding phage, and does not represent a soluble "lysin".

3. The activity of S.S.S. is reduced by heating to temperatures above 65°, and is completely lost after 30 minutes' heating at 90°–93°.

4. By the addition of relatively large amounts of S.S.S. to phage-antiphage mixtures the process of neutralization can be stopped and to some degree reversed.

5. Rabbits inoculated with ultra-filtrates containing S.S.S. show a slight appearance of phage-inactivating antibodies.

I am indebted to Dr. W. J. Elford for carrying out numerous filtrations, and to him and Dr. C. H. Andrewes for much helpful discussion on phage problems.

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STUDIES ON *CLOSTRIDIUM CHAUVŒI*. III.—PASSIVE PROTECTION WITH PURE "O" SERA.

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IN a previous paper (Henderson, 1932, *a*) the identity of the "O" antigen of *C. chauvœi* in strains of bovine and ovine origin from various parts of the world was established by *in vitro* experiment. Active immunization experiments with guinea-pigs corroborated this evidence and showed the high prophylactic value of this antigen (Henderson, 1932, *b*).

The use of such antigens, consisting of washed and steamed bacillary bodies, has been extended to the production of pure "O" sera, and the present paper records the results of passive protection experiments with mice, using pure "O" sera obtained from goats.

PREPARATIONS OF GOAT SERA.

The " O " antigens used for immunization were prepared from 1 per cent. glucose broth growths, twice washed and resuspended in distilled water to an opacity ten times greater than the " Wellcome " standard opacity tube No. 10, and steamed for 2 hours at 100° C. After heating, 10 per cent. sodium chloride was added to give a final concentration of 0·85 per cent. NaCl.

Two goats were immunized. Goat 1 received a vaccine prepared from an English bovine strain (G1), and Goat 2 a vaccine from an English ovine strain (M7). From each goat an adequate supply of normal serum was first obtained, and stored for use as control material in the experiments with immune sera.

The course of immunization extended over four months, and inoculations were made intravenously, approximately twice per week, with two rest periods of 14 and 21 days respectively. Each goat received an amount of antigen equivalent to the growth product of 6 litres of media. The sera obtained were passed through coarse Seitz filters and stored without preservative at $\pm$ 4° C. By the use of the term pure " O " sera it is implied that they were devoid of antitoxin and " H " agglutinin content. The latter fact was established in repeated agglutination tests. While the absence of antitoxin could not be proved experimentally, it is known that no specific soluble toxin has yet been clearly demonstrated in *C. chauvœi*, and it is justifiable to assume that any such hypothetical toxin or " aggressin " would not withstand heating for 2 hours at 100° C.—a treatment to which the antigen has been subjected.

METHOD OF EXPERIMENT.

The method of experiment was similar to that employed by Robertson and Felix (1930) in their experiments with *V. septique*.

Mice of approximately 20–25 gm. were used. The immune serum under test was inoculated intra-muscularly in the left leg, in 1 c.c. quantities, 24 hours previous to testing with the infecting suspension. Simultaneously, a series of mice, to act as controls, received 1 c.c. of the corresponding normal goat-serum.

The infecting suspensions were prepared from the English bovine strain G1 grown and standardized as previously described (Henderson, 1932, *b*) to an opacity five times greater than " Wellcome " standard opacity tube No. 10. The suspensions were freshly prepared for each experiment, and were diluted with saline as required.

The test dose for all mice was given intramuscularly in the leg opposite to the site of serum treatment, in a total volume of 0·7 c.c., and contained 0·3 c.c. of the infecting suspension in the required dilution, 0·15 c.c. freshly prepared 5 per cent. $CaCl_2$ in distilled water and 0·25 c.c. saline.

In each experiment the following groups of mice were included :

1. Mice treated with immune serum receiving the infecting suspension (opacity 10 $\times$ 5) in dilutions from 1 : 100,000 to 1 : 10,000,000.

2. Mice treated with normal serum receiving the test dose in the maximum and minimum opacities used.

3. Norman mice receiving the test doses used and higher dilutions of them to give an indication of the infecting power of the suspension.

4. Normal mice receiving the infecting suspension in the maximum opacity used, without $CaCl_2$ as activator.

5. Normal mice and mice treated with immune and normal serum respectively, receiving the $CaCl_2$ activator alone.

THE EFFECT OF THE SERUM USED.

Table I summarizes the results of one experiment, using the serum prepared from Goat 2 immunized with the English ovine strain M7. The infecting suspension was prepared from the English bovine strain G1, *i. e.* from a heterologous strain.

The limit of the certain infecting power of this suspension was 1 : 10,000,000. Regular deaths occurred in all control mice up to this level, while of 5 mice receiving the highest dilution, 4 survived. The mice treated with normal serum all died, and showed no prolongation of life as compared with normal mice. Calcium chloride and suspension controls were included, but are not shown in the table.

TABLE I.—*Passive Protection in Mice. "O" Serum from Goat 2 (Ovine Strain M7). Infecting Suspension from Bovine Strain G1 (Opacity 10 × 5).*

Dilution of infecting suspension.	Immune serum-treated mice. Number of mice— Tested.	Survived.	Time of death in mice that succumbed.	Control mice. Number of mice— Tested.	Survived.	Time of death in mice that succumbed.
1 : 100,000	14	6	36–40 hrs.	14 (6+N.S.)*	0	18–26 hrs.
1 : 1,000,000	14	8	44–48 ,,	8	0	24–30 ,,
1 : 10,000,000	11	8	44–72 ,,	11 (3+N.S.)	0	30–48 ,,
1 : 100,000,000	..	..	...	5 (2+N.S.)	4	48 ,,

* (No. + N.S.) = Number of control mice receiving 1·0 c.c. normal serum.

The effect of the immune serum is clearly shown. Of 14 mice receiving the lowest dilution of the infecting suspension, 6 survived, of 14 given the intermediate dilution 8 survived, and of 11 receiving the highest dilution 8 survived Further, as shown in Table I, the treated mice which ultimately succumbed did so, without exception, after a longer survival period than the control mice.

A typical reaction to infection in normal mice was the appearance of a marked swelling at the site of inoculation, with or without a blackening of the limb, leading quickly to a perforating gangrene, which regularly was followed by death in from 18–48 hours after infection.

Immune serum-treated mice frequently showed a similar reaction, followed by a perforating gangrene in from 18–48 hours ; a proportion of these mice succumbed while the remainder recovered completely.

Similar experiments were conducted with the serum from Goat 1 immunized

with the English bovine strain G1. In this case the infecting suspension was prepared from the homologous strain. While protective power was clearly established, this serum was definitely less potent than that from Goat 2. Approximately 50 per cent. of mice treated with Goat 1 serum survived the smallest certain infecting dose of the test suspension, while with Goat 2 serum approximately 70 per cent. of mice survived. With an infecting dose ten times greater 25 per cent. of mice receiving Goat 1 serum survived, while Goat 2 serum gave a 55–60 per cent. survival rate.

"O" AGGLUTININ CONTENT OF SERA OF IMMUNIZED GOATS.

In view of the variation in the protective power of the two immune sera, a comparison of their behaviour in "O" agglutination is of interest. The immune serum from Goat 2 with the higher protective value contained "O" agglutinins to a titre of 1 : 1250 to 1 : 1500 for both homologous (M7) and heterologous (G1) strains. The immune serum from Goat 1 on the other hand immunized with strain G1 contained "O" agglutinins to a titre of 1 : 1000 for both strains. The corresponding normal sera from the two goats in a dilution of 1 : 10 contained no agglutinins for either strain. The higher "O" titre of the immune serum from Goat 2 thus reflects its higher protective value

DISCUSSION.

The objective in this work and in the previously reported experiments on active immunization of guinea-pigs has been to demonstrate the *rôle* of anti-bacterial mechanisms in *C. chauvœi* prophylaxis. From the results of the earlier work (Henderson, 1932, *b*) it was shown that the "O" antigen was a powerful immunizing agent, and it was suggested that the formolized whole culture vaccines and the bacteria-free "aggressins" widely used in *C. chauvœi* prophylaxis owe their immunizing property to the "O" antigen which they contain. The experiments now reported show the anti-bacterial power of an "O" immune serum in passive protection, and the results obtained corroborate the previous findings.

While passive and active protection cannot easily be compared, it would appear that the protective power of the "O" sera used in these experiments with mice is appreciably lower than that previously demonstrated by active immunization of guinea-pigs. In this connection the following points should be considered:

(*a*) No attempt was made to produce sera of maximum potency, the intention being primarily to obtain evidence of passive protection. There is little doubt that by continuing the immunizing process, or by separating concentrated globulin fractions from the sera, their protective value could have been raised.

(*b*) Goats were chosen for the production of "O" sera as a matter of convenience. There is no indication that a more suitable animal could not have been selected.

(*c*) The protection afforded compares very favourably with that conferred by commercial horse-serum produced by the use of formolized whole culture or "aggressin" (McEwen and Roberts, 1932; Scott, 1932).

The comparison of the " O " agglutinin content of the two goat sera would indicate that their relative value in passive protection is expressed in their " O " agglutinin titre. The evidence suggests that the " O " antigen functioning in agglutination is also responsible for the production of the protecting immune body.

This brings confirmatory evidence to the view, now more generally accepted, that the " O " antigen and its corresponding antibody are the important factors in antibacterial mechanisms in the animal body.

SUMMARY.

Passive protection experiments with mice are described, using pure " O " sera prepared from two goats.

The relative protective values of the two sera are discussed and their relation to " O " agglutination is considered.

The value of antibacterial mechanisms in *C. chauvœi* prophylaxis is discussed.

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A VITAMIN NECESSARY FOR THE GROWTH OF *B. SPOROGENES*: ITS RELATION TO AUXIN AND OTHER GROWTH FACTORS.

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IN a communication to the Physiological Society (Knight and Fildes, 1933) we described shortly a factor essential for the growth of *B. sporogenes*. In the present paper we give fuller details. This sporogenes factor we look upon as a vitamin, using this expression in the sense in which it is used in animal physiology, namely, a substance necessary for growth, but active in concentrations so small that its cannot, in the light of present knowledge, be credited with supplying significant amounts of energy or of constructive material.

We separated this factor while fractionating various extracts in the course of a study of the food requirements of *B. sporogenes*, and in order to demonstrate its existence it is necessary to evolve a diet for *B. sporogenes* which does not contain this factor, but is otherwise adequate for growth.

* Both working with grants from the Medical Research Council.

TECHNICAL DETAILS.

(a) *The vitamin deficient medium.*—The various ingredients sufficient for one tube of medium are mixed after separate sterilization in bulk in the autoclave :

1. Sodium citrate, 2·0 gm. ; $MgSO_4 7H_2O$, 0·5 gm. ; M/15 phosphate buffer, pH 7·4, 500 c.c. autoclaved .	5·0 c.c.
2. Nelson's photographic gelatine No. 1, 200 gm. dissolved in 1000 c.c. 10 per cent. H_2SO_4 and autoclaved for 3 hours at 130° C. Remove sulphates with baryta and evaporate to about 400 c.c. as stock. For use dilute with distilled water to make equivalent to 5 per cent. gelatine ; autoclaved	1·0 ,,
3. Tryptophane 0·1 per cent., autoclaved . . .	0·2 ,,
4. Thioglycollic acid 1 per cent. in N/1 HCl, autoclaved .	0·2 ,,
5. N/1 NaOH in water, autoclaved	0·25 ,,
6. Water, autoclaved	2·35 ,,
	9·00 c.c.

Solutions to be tested for vitamin activity were added in a bulk of 1 c.c. as serial dilutions in distilled water. Water was used as a control.

(b) *The inoculum.—B. sporogenes* (No. 533 of the National Collection of Type Cultures) grown for 4 days on agar surfaces ; washed off with distilled water ; filtered through glass wool ; heated to 65° C. for 1½ hours ; centrifuged and washed 4 times with distilled water. One and the same suspension was used during the whole work lasting 3 years. One drop of the stock suspension was diluted with 10 c.c. distilled water, and of this 1 drop was inoculated. The approximate number of spores in the inoculum was 11,000 as a routine procedure, but growth has been obtained from an inoculum calculated to contain 3 spores.

(c) *Purity of constituents of the medium.*—All chemicals were of the finest commercial grade and re-crystallized at least 3 times. Thioglycollic acid was used as received. Water was freshly distilled and autoclaved.

(d) *Culture vessels, etc.*—All glass-ware was cleaned in 66 per cent. H_2SO_4 saturated with bichromate. " Monax " test-tubes, 6 × ¾ in., plugged with white wool covered with washed gauze.

(e) *Incubation* at 38° C. in McIntosh and Fildes's electric jar.

(f) *Reading results.*—Growth was judged by opacity. In the presence of the vitamin in sufficient concentration, full growth is obtained in 17–20 hours, but 40–48 hours is required to disclose growth n low concentrations. The mass of growth is not as great as that seen in broth, for instance, but no effort has been made to increase the mass of growth by manipulation of sources of energy, etc.

(g) *The control* containing no vitamin should, under ideal conditions, of course, give no growth, but in practice minute traces are observed with some preparations of gelatine. However long incubation is prolonged these traces

do not increase. We have found no method which can remove the traces of vitamin in gelatine without destroying other essential properties.

The outstanding object of these details is to obtain a vitamin-free diet upon which the activity of the vitamin preparations can be tested. Owing to the presence of this substance in organic matter generally (for instance, cotton-wool) in sufficient amount to interfere with the tests, it is essential to avoid inadvertent contamination with it. The gelatine fraction of our medium is, however, sometimes a source of traces which cannot be excluded. Subject to the qualification required by these occasional traces, we emphasize that the sporogenes vitamin is a substance which makes the difference between no growth and growth; it is not a mere stimulant of a feebler growth which can take place without it. The growth is proportional to the amount of vitamin added up to the point of "full growth". Further addition then makes no difference, presumably because some other of the factors involved becomes limiting. The growth is, of course, capable of being propagated indefinitely in series in the same medium.

THE PREPARATION OF THE ACTIVE SUBSTANCE FROM YEAST.

Extracts can be obtained from many sources which, as a supplement to the inadequate basal medium described above, will enable *B. sporogenes* to grow. For convenience we chose pressed baker's yeast, although this may not be the richest source.

Table I shows the steps we used to obtain an active preparation from yeast, omitting the steps that were abortive.

The following experimental notes amplify the table: The yeast was dried at 50° C. and ground to a fine powder. This was extracted with successive quantities of 75 per cent. aqueous ethyl alcohol, containing 0·5 per cent. H_2SO_4, by boiling under a reflux condenser. Usually 6–7 extractions were made, using the equivalent of 1500–2000 c.c. lots of extracting liquid per kilogram of dried yeast.

The alcoholic extract (A) was concentrated at 60–70° C., initially by distillation under reduced pressure, and finally in an open dish on a water-bath at 70°, accelerating the evaporation by stirring and the use of an electric fan. The concentrated extract was a greasy, yellow mass of treacly consistency. Plaster-of-paris was stirred into this while still hot, to give a dry mass suitable for Soxhlet extraction.

The acetone precipitate from the ether extract (B) was re-dissolved and re-precipitated to sharpen the separation.

The acetone-ether soluble lipoidal material (C) after removal of the solvent was dissolved in ethyl alcohol, and saponified by refluxing for 3 hours with aqueous 5 per cent. NaOH.

The ether extractions from alkaline, bicarbonate and acid aqueous solutions were straightforward. The distribution of activity in these three stages is illustrated in Table I. It will be noticed that the separations were sharp except during extraction from aqueous acid, when slight activity remained in the aqueous layer.

The ether extractions established that the active substance is acidic, and

TABLE I.—*Concentration of Sporogenes Vitamin from Yeast.*

YEAST

Dried and extracted with boiling 75 per cent. aqueous ethyl alcohol

EXTRACT A

Dried with plaster-of-paris and ether extracted in Soxhlet

ETHER EXTRACT B (ACTIVE)

Ether solution precipitated with acetone

Precipitate ether-acetone insoluble (inactive)	ETHER-ACETONE SOLUBLE C (ACTIVE)
	Saponification and ether extraction
Ether extract (inactive)	AQUEOUS NaOH D (ACTIVE)
	Saturated with CO_2 and ether extracted
Ether extract (inactive)	AQUEOUS BICARBONATE E (ACTIVE)
	Acidified H_2SO_4 and ether extracted
Acid aqueous fraction, only slight activity	ETHER-SOLUBLE FRACTION F ORGANIC ACIDS (ACTIVE)
	Barium precipitation from dilute aqueous alcohol
Precipitated Ba salts	SOLUBLE BA SALTS (ACTIVE)
	Ba removed with dilute H_2SO_4
Free acids (inactive).	FREE ACIDS: LIGHT YELLOW GUM G, 1/5,000,000 gm. activates 10 c.c. of basal medium.

that it is fairly strongly polar, since the partition coefficient ether/water is not large.

The organic acid fraction (F) contained fatty acids, which, giving soaps in the culture medium, tended to inhibit growth at the higher concentrations.

After trying several methods it was found that this organic acid fraction dissolved in aqueous alcohol gave with baryta a precipitate of barium soaps, leaving the barium salt of the active substance in solution. The filtrate was exactly freed from barium by dilute sulphuric acid, the barium sulphate removed, and the filtrate evaporated at low temperature with the addition of ethyl alcohol.

This active product was a yellow gum (G), and $2{\cdot}0 \times 10^{-7}$ gm. of it activated 10 c.c. of the basal medium, giving a growth just visible to the naked eye.

Chemical Tests on the Active Yeast Preparation.

The following are the results of chemical tests on the gum G at this stage: nitrogen, sulphur and halogens absent (25 mgm. fused with sodium).

A 0·5 per cent. solution of the gum G in water was acid to litmus, yellow and faintly opalescent, giving a clear solution on adding a little alcohol or alkali. The opalescence vanished, and the solution became a brighter yellow on making alkaline with baryta. The 0·5 per cent. solution gave the following tests:

(i) Alkaline K MnO_4: Easily reduced at room temperature.
Neutral K MnO_4: Reduced at room temperature.
Acid K MnO_4: Reduced, but more slowly than alkaline at room temperature.

(ii) Ferric chloride: Yellow colour; more intense orange colour on making alkaline; $Fe(OH)_3$ only precipitated after some time.

(iii) Fehling's solution: Not reduced, even after hydrolysis with dilute H_2SO_4.

(iv) Nitric acid: On warming and making alkaline with ammonia gave a bright yellow colour.

(v) Millon reaction: Positive.

(vi) Molisch, glyoxylic and biuret tests: negative.

It does not follow that the positive reactions are due to the active substance. But evidence that this may be so is provided by the following comparisons: 10 c.c. of the 0·5 per cent. solution of G were made alkaline with NaOH, and saturated with CO_2 and extracted with peroxide-free ether (6×10 c.c.). Ether was removed from both fractions, and each made up to 100 c.c. after making neutral to litmus. Portions of each fraction were then tested.

	$NaHCO_3$ fraction.	Ether fraction.
Millon	Positive.	Negative.
Acid $KMnO_4$	Rapid reduction.	Very slow reduction.
Alkaline $KMnO_4$	About same rate in each.	
$FeCl_3$	Brighter yellow.	No change.
Addition of NaOH	$Fe(OH)_3$ not precipitated.	$Fe(OH)_3$ precipitated at once
Growth test	Bicarbonate fraction 25 times more active than ether fraction.	

A similar test was made on the distribution of activity and chemical reactions between ether and dilute sulphuric acid. 10 c.c. of the 0·5 per cent. solution of G were acidified with H_2SO_4 and ether extracted (6 × 10 c.c.). The two fractions were freed from ether, neutralized to litmus and made up to 100 c.c. Portions of each fraction were then tested.

	Ether fraction.	Dilute H_2SO_4 fraction.
Xanthoproteic	Positive.	Negative.
Millon	,,	,,
Alkaline $KMnO_4$	Rapid reduction.	Slow reduction.
Acid $KMnO_4$	Rapid reduction ; uses at least 10 times more $KMnO_4$ than the other fraction.	Very slow reduction, if any, with 2 drops.
Growth test	5–10 times more active.	..

From these comparisons of the distribution of the activity and chemical reactions it is evident that the active substance, the positive Millon reaction and the easy reduction of permanganate go hand in hand. The distribution of the substance giving the positive Millon reaction indicates that it may be a phenolic acid. It remains to be seen whether this parallelism continues on further purification. The effect of oxidation by H_2O_2 on the activity was tested. 1 c.c. of 0·5 per cent. solution of G was warmed with 5 c.c. of 10 vol. H_2O_2 and allowed to stand 2 hours. Excess of H_2O_2 was then decomposed by making alkaline with NaOH and warming. The solution was made up to known volume and tested for activity. This was found to have been lost.

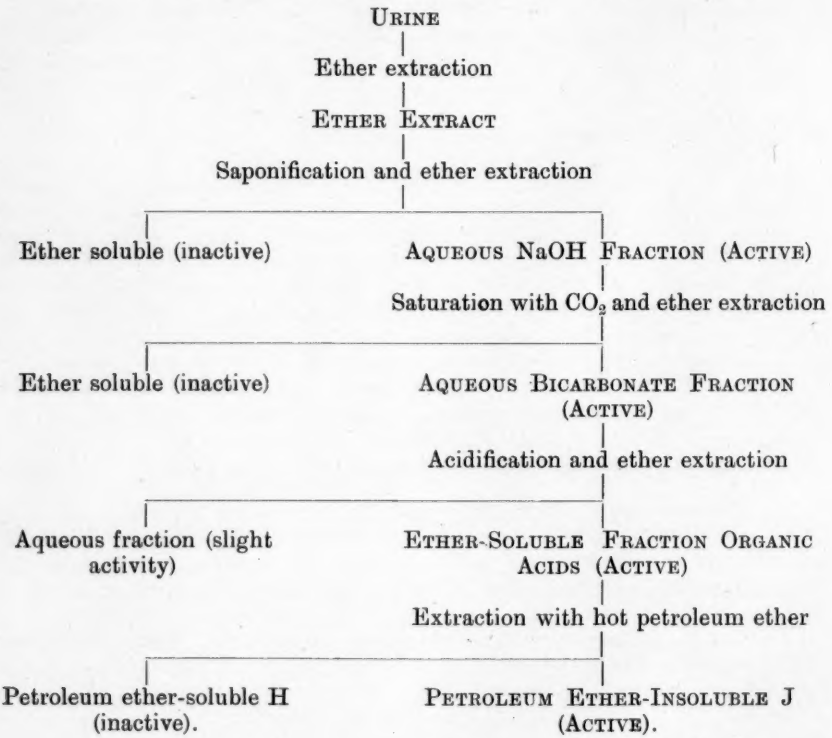
THE PREPARATION OF THE ACTIVE SUBSTANCE FROM URINE.

At this stage we recognized the similarity between our substance and auxin (Kögl and Haagen Smit, 1931 ; Kögl, 1932, 1933) which has to do with stimulating the elongation of plant-cells. Normal human urine has been found to be a convenient source of auxin, and consequently we examined urine for the sporogenes factor.

Two litres of male urine (Table II) were concentrated under reduced pressure and at a temperature less than 60° C. The concentrate (about 300 c.c.) was acidified with H_2SO_4 and extracted with peroxide-free ether. There was a tendency to give stable emulsions, which could be largely avoided by very gently shaking. The ether was removed from the extract and the residue saponified by heating with 200 c.c. 5 per cent. aqueous NaOH for 4 hours. The saponification mixture was ether-extracted in the usual three stages : alkaline, saturated with CO_2, acidified with H_2SO_4. The three extracts and the aqueous residue were freed from ether, made up to a suitable volume in water and tested for activity. The distribution of activity is shown in Table II, which is a composite of all the experiments with urine. The distribution of activity again indicated an organic acid. The phenolic fraction which was inactive would contain trihydroxyœstrin, ketohydroxyœstrin, and pregnandiol, and pure samples of these compounds, supplied by Dr. G. F. Marrian, showed no activity on *B. sporogenes*.

These experiments show that urine provides a very convenient source of the sporogenes factor. Since the bicarbonate fraction which contains the factor is a by-product in the commercial preparation of the œstrin compounds from pregnancy urine, large quantities of this are readily available. A sample of this fraction supplied by British Drug Houses, Ltd. (U_1, B.D.H.), contained 0·247 gm. solid per c.c. (dried on a water-bath). The minimum growth dose

TABLE II.—*Concentration of Sporogenes Vitamin from Urine.*



was 0·00005 c.c., equivalent to $1{\cdot}23 \times 10^{-5}$ gm. per 10 c.c. of medium. We have not compared the activity of male, female and pregnancy urines, but all contain the sporogenes factor and auxin. Acidification of U_1, B.D.H., with sulphuric acid caused a tar and some precipitate to separate, which made the ether extraction difficult. After one ether extraction the aqueous liquor was filtered and the precipitate washed with alcoholic ether. Ether extraction of the aqueous filtrate was continued. Finally the total (alcoholic) ether extract, after suitable washing, was shaken with three successive 100 c.c. lots of dilute NaOH. This soda solution was dark red-brown in colour. This was

acidified with H_2SO_4 and, ether extracted, the extract washed once with distilled water and the solvent removed. The residue was a dark brown oil, setting to a semi-crystalline mass on cooling. This contains about 75 per cent. benzoic acid. This solid residue was boiled with three successive quantities of petroleum ether, in which benzoic acid is soluble. The petroleum ether-soluble fraction (H) and the insoluble residue (J) were tested for activity. The activity was in the petroleum ether-insoluble fraction, as Kögl finds for auxin. The urine preparation is at present not so active as the yeast preparation, and the latter is much less pigmented. But since the concentrated bicarbonate fraction of the ether-soluble part of urine is so readily available, this is undoubtedly the best source for future work. There were considerable losses of activity in the separations, which we have not yet traced. We were concerned in this first instance to concentrate the sporogenes factor, and to compare its distribution, etc., with that of auxin. All the methods we have used for concentrating the sporogenes vitamin have included an alkaline saponification. Kögl (1933) finds that alkaline saponification of auxin esters yields an inactive product. In all other stages of the separations the sporogenes vitamin behaves in its distribution between solvents as would auxin.

Since the distribution coefficient between acid-water and ether is not very favourable for rapid ether extraction we have tried other methods. Extraction of the diluted bicarbonate fraction, after acidification with H_2SO_4, by butyl alcohol, and subsequent extraction of the butyl alcohol with sodium bicarbonate solution, yields a sharper separation, and much pigment is left behind in the acid aqueous and butyl alcohol fractions. There is, however, a considerable loss of total activity. Probably one of the reasons for this is destruction of activity by oxidation, and precautions must be taken to avoid this as much as possible. We find that ether containing some ethyl alcohol is a better extracting liquid than pure ether.

COMPARISON BETWEEN AUXIN AND THE SPOROGENES VITAMIN.

The resemblances between the properties of auxin and the sporogenes vitamin so far as the latter are known may be summarized :

Both auxin and the sporogenes vitamin occur in urine.

The further stages of the method which Kögl has used to isolate auxin have

Auxin.	Sporogenes vitamin.
Monocarboxylic acid.	Acidic substance.
Soluble in ether.	Soluble in ether.
Insoluble in petroleum ether.	Insoluble in petroleum ether.
Calcium salt soluble in aqueous alcohol.	Barium salt soluble in aqueous alcohol.
Contains C, H and O only.	Does not contain N, S, or halogens.
$KMnO_4$ gives a C_{13} dicarboxylic acid. Contains one double bond.	Easily oxidized by $KMnO_4$ and H_2O_2.
Oxidation destroys activity.	Oxidation destroys activity.
Contains 3 hydroxy groups, and possibly a carbon ring.	Millon positive, $FeCl_3$ yellow.

not yet been carried out for the sporogenes vitamin. The crucial experiment of testing pure auxin on *B. sporogenes* must await our obtaining a sample of this compound.

It is evident from the distribution and properties of the sporogenes vitamin that, so far as it is yet purified, it shows a striking relation to auxin.

SYNTHESIS OF SPOROGENES VITAMIN BY BACTERIA AND MOULDS.

Active extracts containing the sporogenes vitamin can be obtained from many sources—animal tissues and fluids, yeast, urine, in fact from almost any living material. Five times recrystallized egg-albumin contained it. The first two or three crystallizations did somewhat reduce the amount, but the subsequent crystallizations effected no improvement.

It is important, therefore, to discover where the vitamin is synthesized. As is well known, some species of bacteria can grow without difficulty on artificial media containing only a few simple compounds, *e. g.* citrate, ammonium chloride and the usual salts, and the question arises whether these types of bacteria really grow without the sporogenes vitamin or whether they can synthesize it.

We grew *B. œrtrycke* in a medium containing only sodium citrate, ammonium chloride, $MgSO_4$ and phosphate buffer, and were supplied with a flask of *B. tuberculosis* 100 by Capt. S. R. Douglas grown on asparagine, glycine, guanidine hydrochloride, glycerol, citrate, ammonium chloride and the usual salts.

Controls showed that the media themselves did not contain the sporogenes vitamin. In both cases the inocula were from growths which had been cultivated on their respective media for many subcultures. The possibility that sufficient sporogenes vitamin might be introduced in the inoculum to account for the subsequent growth therefore seems to be ruled out. The cutures were made alkaline (N/20 soda), autoclaved, filtered through a Seitz EK, and tested, after neutralization, for the sporogenes vitamin. In both cases a positive result was obtained, showing that these two organisms had synthesized the vitamin. These two organisms were chosen at random from among those able to grow without difficulty on purified synthetic media.

We found that the sporogenes vitamin was synthesized also by a mould growing on a synthetic medium. Prof. J. C. Drummond and Mr. R. L. Edwards supplied us with preparations from *Aspergillus versicolor* grown on a variety of carbon sources. The latter were acetate, tartrate, mannitol, glucose, stearic acid and paraffin, and in all cases the sporogenes vitamin was synthesized.

DISCUSSION.

The Vitamin Conception in Medical Bacteriology.

The idea that bacteria, especially those of medical interest, require for their growth vitamins or "accessory growth factors" has been advanced for some years, and some authors have claimed to have proved the necessity for this requirement. The literature has been surveyed by Knorr (1925), Sergent

(1928) and Peskett (1933). In our view many authors have been led into error in the conclusions they have drawn. For instance, Hosoya and Kuroya (1923) have been quoted frequently as having demonstrated the necessity of a vitamin for a large number of bacteria. We find this claim to be unfounded for many reasons, of which we will mention one. These authors arbitrarily selected a certain preparation of "Vitamin B" as their vitamin, and demonstrated that this substance in a concentration of 0·05 per cent. in an inadequate medium caused growth of streptococci and other organisms which failed to grow in the controls. Their "Vitamin B" gave negative colour tests for proteins and amino-acids and was, therefore, assumed to be free from such bodies, the growth being ascribed to the "vitamin" content. To illustrate the fallacy of this argument we may remark that *B. sporogenes*, provided with sporogenes vitamin and the other ingredients of our deficient medium except tryptophane, will still grow, although if the gelatine hydrolysate is precipitated with mercuric sulphate according to the Hopkins procedure for removing tryptophane, no growth occurs. Thus tryptophane is an essential for the growth of *B. sporogenes*, and will cause full growth in a concentration very much smaller than that of the "vitamin" used by Hosoya and Kuroya. Further, the amount of tryptophane present in gelatine, though adequate for growth, is not demonstrable by the glyoxylic test until the mercury precipitate has been concentrated to an extent equivalent to a 100 per cent. solution of gelatine. The absence of a colour test, therefore, does not exclude the presence of a foodstuff in amounts significant in this type of work. Broom (1929) has already exploded the argument that initiation of growth in a deficient medium must be due to a vitamin by showing that the effects ascribed to vitamins in orange-juice were due to glucose and other fermentable carbon compounds therein. We are therefore unable to accept the claims of Hosoya and Kuroya, nor for similar reasons the claims of other workers.

There are, however, two reports in the literature of the separation of fractions highly active for bacteria. Mueller (1920, 1922, 1923) separated a fraction from casein which would activate a medium deficient for hæmolytic streptococci in a concentration of 0·004 mgm. per 10 c.c. The chemical nature was not determined. Hughes (1932) obtained a fraction from meat extract thought to be a natural base which would activate a casein hydrolysate for staphylococci in a concentration of 0·0002 mg. per 10 c.c., *i. e.* a concentration comparable to that in which our present specimen of sporogenes vitamin is active.

Whether our vitamin is active for bacteria other than *B. sporogenes* we cannot at present say. Neither *B. tetani* nor *B. œdematis maligni* will grow upon the vitamin-containing medium we have used for *B. sporogenes*, but it is at least possible that this failure is not due to the inadequacy of the vitamin, but of other ingredients of the basal deficient medium. Any extract or preparation which is capable of growing these two organisms already contains the sporogenes vitamin, and therefore cannot be used as a "deficient" medium to demonstrate its effect.

There is, however, evidence, direct and indirect, which is detailed later, which shows that the activity of our sporogenes vitamin is not confined to *B. sporogenes*.

The Relation of Sporogenes Vitamin to Other Growth Factors.

In a previous section are recorded the data showing the chemical similarities between auxin and the sporogenes vitamin, but when their physiological effects are compared they appear to be different.

Kögl (1933) specifically states that auxin has to do with plant-cell elongation and not cell multiplication. He also reports that "auxin has nothing to do with 'bios', the growth activator of yeast and other micro-organisms". Actually the latter is a statement very difficult to prove. The accumulated evidence of yeast "bios" work indicates the "bios" is not an entity, and that different yeasts have different "bios" requirements. No details are given of the experiments that were made to test the possible "bios-activity" of auxin. But the fact that auxin was apparently not active under the conditions used does not necessarily exclude the possibility that it might be active under other conditions. For instance, a yeast may have been chosen which required some other factor as well. This we know to be possible, since Edwards (1933) has a preparation active for Gebrüder Mayer yeast, and not active for a strain (2190) of *Saccharomyces cerevisiæ*. Also, our sporogenes vitamin preparation tested by Mr. Edwards was active for Gebrüder Mayer yeast only.

The facts that auxin under the conditions of test stimulates plant-cell elongation but not division, whereas the sporogenes vitamin causes "growth" —a complex of elongation and division—do not necessarily imply a fundamental difference in behaviour.

The method of studying the physiological activity of preparations stimulating plant-cell elongation has been standardized for quantitative measurement by Went (1928) and van der Wey (1931). Oats, *Avena sativa*, are allowed to germinate and the coleoptiles are decapitated. The substance to be tested is applied in a cube of agar one-sidedly to the cut surface of a decapitated coleoptile. The cells on the side on which the cube stands are stimulated to elongate more than those on the other side, and the coleoptile bends.

The method of testing is a relatively static one, the growing tip of the coleoptile where the cells would be dividing having been removed; in this way one phase of the growth process is conveniently isolated. It seems to us therefore that an effect of auxin upon cell multiplication cannot be excluded until its effect has been studied under conditions where there would be opportunity for multiplication to occur. With free cells, such as bacteria and yeasts, the effect of growth factors on different phases of the growth process cannot be so easily separated as in the linear growth of a coleoptile. But the elongation and swelling of bacteria as a preliminary stage in division is a general phenomenon, and may be, indeed, a pre-requisite to normal division.

We think, therefore, that even if our sporogenes vitamin is not identical with auxin, they may both be members of a related group of substances intimately concerned with the regulation of the growth process in plants, bacteria, yeasts and moulds. Evidence supporting this generalization is given by the following facts: As we have shown, the sporogenes vitamin is synthesized by two quite distinct bacteria, *B. aertrycke* and *B. tuberculosis*, and by a mould, *Aspergillus versicolor*. These are the only micro-organisms we have as yet tested, but we are confident that others capable of growth on simple synthetic

media will be found to synthesize the sporogenes vitamin. We do not think the factor we have distinguished can be *specific* for *B. sporogenes*. It is highly improbable that three widely different micro-organisms, chosen haphazard from among those capable of growing without difficulty on simple synthetic media, should all synthesize a substance required by *B. sporogenes* only.

There is the further evidence that our preparation can act as " bios " for Gebrüder Mayer yeast, although not for Edwards's strain 2190 of *Saccharomyces cerevisiæ*. *Aspergillus versicolor* grown on synthetic medium with various sources of carbon synthesized material active for *Saccharomyces cerevisiæ* 2190 and Gebrüder Mayer, with the exception that on acetate the mould produced a factor active for Gebrüder Mayer only and not for 2190 (Edwards, 1933). On this same carbon source, as well as on the others, the sporogenes vitamin was produced. These results seem to indicate a relation between the Gebrüder Mayer factor and the sporogenes vitamin. The Gebrüder Mayer yeast which Mr. Edwards uses was obtained from R. J. Williams's laboratory. It is for this yeast that Williams and co-workers (1932) claim to have isolated a highly active polyhydroxy acid. They find this widely distributed in living material, including *B. subtilis* cultures. Its chemical properties relate it to the sporogenes vitamin and to auxin. Other evidence for the existence of this suggested group of substances is provided by the work of Schopmeyer and Fulmer (1929) and Schopmeyer (1931) on a growth stimulant for *Saccharomyces cerevisiæ*, which is alcohol-ether soluble. This was obtained from synthetic cultures of various moulds : *Aspergillus niger*, *A. clavatus*, *Trichoderma lignorum* and *Rhizopus nigricans*.

More recently Philipson (1933) has partially separated an active factor for various yeasts from green peas, which is also alcohol-ether soluble. Guha (1932) records the synthesis of " bios " for *Saccharomyces cerevisiæ* by *B. vulgatus* (Flügge) grown on synthetic medium, but did not determine solubility differences of the factor.

The distribution of auxin, or of related substances effecting cell elongation in Avena coleoptiles, is also highly suggestive. This substance or group of related substances is produced by moulds (Nielsen, 1931, 1932 ; Nielsen and Hartelius, 1932 ; Kögl and Haagen Smit, 1931 ; Kögl, 1933 ; Boysen-Jensen, 1931, *b*, 1932 ; Dolk and Thimann, 1932) and by bacteria (Boysen-Jensen, 1931, *a* ; Kögl and Haagen Smit, 1931). Most of the moulds were grown on synthetic media. Kögl and Haagen Smit find auxin in cultures of *B. coli*, but do not record whether they were grown on a simple synthetic medium.

There are also converse observations of Euler and Philipson (1932) that Avena coleoptiles yield a growth factor for *Saccharomyces cerevisiæ*, which is also effective in promoting the growth of various moulds.

From a consideration of the above inter-related evidence we think it not unlikely that auxin and the sporogenes vitamin, if not identical, are members of a related group of substances, essential for the growth of bacteria, yeasts, moulds and plants. If any members of these groups of organisms are incapable of synthesizing this substance or group of substances for themselves, then they must be supplied with it in their nutrients. In Nature those organisms incapable of this synthesis depend on the production of the active substance or substances by those able to synthesize it.

CONCLUSIONS.

1. A factor, comparable to a vitamin, necessary for the growth of *B. sporogenes* is described. Its chemical nature is indicated.

2. Evidence is brought forward to suggest that this sporogenes vitamin may be closely allied to other factors which have been described as affecting the growth of plants and other forms of life.

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